REMARKS

Election/ Restriction Requirement and claims currently under consideration

Contrary to the assertion of the Examiner, the election of Group II was made with traverse (at least because a search of one of the indications (such as the indication of Group II) would likely cover the other indications (*e.g.* of groups I, III and IV), as well). Therefore, applicants request that the election of Group II be considered to have been made with traverse.

Furthermore, in view of the Examiner's suggestion that if linking claims 1-2 and 19-80 are found to be allowable, groups I, III and IV would be rejoined, applicants again request that this be done. It is noted that the administration of a polynucleotide encoding VEGF to cardiomyocytes or a tissue that comprises cardiomyocytes (*e.g.* to the myocardium), at a dose that is effective to induce cardiomyogenesis, will also, inherently, result in the indications of groups I, III and IV (induction of arteriogenesis, lymphangiogenesis and vasculogenesis).

In addition to requesting that the withdrawn claims from Groups I, III and IV be rejoined if claims of Group II are found to be allowable, applicants request that withdrawn claims directed to the species which have not been elected be examined if the elected species are found to be free of the prior art of record.

In addition, applicants request that some of the claims which have been withdrawn be examined at this time. For example:

Claims 40 and 41 should not have been withdrawn. These claims recite intravenous or intra-arterial administration of a plasmid. Both of these modes of administration are well-known to result in the transport of a plasmid to, and the transfection of, striated muscle tissue, including myocardial tissue (*e.g.* a cardiomyocyte or tissues comprising cardiomyocytes).

It has been known for many years that naked plasmid DNA can be administered by an intravascular route to deliver the DNA to muscle tissue in the body. See, *e.g.*, Budker *et al.* (1998) *Gene Therapy* 5, 272-276, a copy of which is attached for the convenience of the Examiner, which demonstrates directly the extravasation of DNA that has been injected intravascularly. Several other papers that pre-date the effective filing date of the present application report morphological characteristics of vessels and muscles that support the use of an intravascular route of administration. These papers (which will be provided to the Examiner upon request) include

Browning *et al.* (1996) "Capillary density in skeletal muscle of Wistar rats as a function of muscle weight and body weight", *Microvasc. Res.* 52, 281-7 and Lee *et al.* (1995) "Biomechanics of skeletal muscle capillaries: hemodynamic resistance, endothelial distensibility, and pseudopod formation," *Ann. Biomed. Eng.* 23, 226-46. Furthermore, more recent publications (which will be provided to the Examiner upon request) confirm high levels of expression in muscles following intravascular (artery or venous) injection of naked plasmid DNA. These papers include, *e.g.*, Hagstrom et al. (2004) "A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs," *Mol Ther.* 10, 386-98 and Liang *et al.* (2004) "Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA", *Gene Ther.* 11, 901-8. Furthermore, Wolff *et al.* (2005) "The mechanism of naked DNA uptake and expression." *Adv Genet.* 5, 3-20 summarizes many previous studies and discusses factors involved in efficient transfection of muscle tissues. See, *e.g.*, pages 15 and 16. This reference is attached for the convenience of the Examiner.

Also, claim 42, directed to intracelomic (intrapericardial) administration should not have been withdrawn, since this mode of administration would also result in the administration of a plasmid to cardiomyocytes or tissue comprising cardiomyocytes.

Also, claims 48-50, directed to various forms of administration to heart muscle, should not have been withdrawn, since such administration would also result in the administration of a plasmid to cardiomyocytes or tissue comprising cardiomyocytes.

Also, claims 74, 75, 77, 78 and 80, directed to administration to normoperfused, ischemic or hypoperfused tissue, should not have been withdrawn. Cardiomyocytes or tissue comprising cardiomyocytes "in need of the induction of cardiomyogenesis" include all of these types of tissue, and thus should be examined. Example III in the application (*e.g.* at page 28, lines 1-9) demonstrates, *e.g.*, the administration of a plasmid to an area of injection that included normoperfused tissue, hypoperfused tissue, and the transition zone. Note that the "transition area" includes, *e.g.*, ischemic and hypoperfused tissue. The transition zone is the border area of the ischemic epicenter and comprises the tissue between the area under risk and the normoperfused tissue. Also, it is requested that new claims 103 and 104, which recite administration to hypoperfused tissue, be examined.

Also, claim 76, directed to the induction of tissue regeneration, should not have been withdrawn. Cardiomyogenesis (including mitosis, cell division, and cell proliferation) results in tissue regeneration (*e.g.* reduction in the size of infarcted tissue).

Therefore, it is requested that claims 40-42, 48-50, 74, 75, 77, 78 and 80 (as well as newly added claims 103 and 104) be examined at the present time. These claims are not listed in the current Listing of the Claims as having been withdrawn.

Amendments to the claims

Some of the claims have been amended to address points raised by the Examiner or to clarify aspects of the invention. The amendments are fully supported by the specification and do not add new matter.

For example, some of the claims (*e.g.* claims 1 and 65) have been amended to clarify that the VEGF polypeptide is VEGF-165; that the cells or tissues to which a polynucleotide encoding VEGF is administered is a cardiomyocyte or a tissue comprising cardiomyocytes; and/or that the VEGF coding sequence is operably linked to a CMV promoter and is in a plasmid vector. The recitation that the coding sequence is operably linked to a CMV promoter and is in a plasmid vector is supported throughout the specification. For example, the Examples show the use of pUVEK15VEGF, a vector in which VEGF coding sequences are under the control of a CMV promoter.

Claims directed to the restricted groups other than Groups I, II, III and IV (*e.g.*, claims 81-97) have been canceled.

Claims which have been categorized by the Examiner as falling into restriction Groups I, III or IV have been withdrawn by the Examiner. However, as discussed elsewhere in this Reply, it is requested that those claims be rejoined to the claims from Group II, once those claims have been found to be allowable.

Claims 40-42, 48-50, 74, 75, 77, 78 and 80 are directed to methods of administration that applicants argue elsewhere in this Reply will result in the delivery of the claimed plasmid to cardiomyocytes or tissues comprising cardiomyocytes. These claims were initially withdrawn by the

Examiner. However, because applicants believe these claims should not have been withdrawn, these claims are not listed as having been withdrawn in the current Listing of the Claims.

Claims directed to the administration of the claimed plasmid to cells or tissues other than cardiomyocytes or tissues that comprise cardiomyocytes have been canceled (*e.g.*, claims 15-18, 67-68 and 70).

New claims 98 and 99 recite the amount of polynucleotide that is administered in terms of mg/kg. An amount of a VEGF plasmid of about 0.04 mg/kg is equivalent to about 0.0165 nmole/kg. Support for the recitation of "greater than about 0.04 mg/kg" is found, *e.g.*, in the Examples, in which pigs (which, at the time of analysis - the second surgery - are about 45 kg) are treated with a total dose of about 3.8 mg of VEGF plasmid.

New claim 102 is supported in the specification, *e.g.* at page 9, lines 17-27.

New claims 103 and 104 are supported in the specification, *e.g.* at page 13, lines 21-24. Note that this passage indicates that hypoperfused tissue may include ischemic, viable, hibernated, stunned, preconditioned, injured, infarcted, non-viable, fibrosed or necrosed tissue.

Claims 15-18, 28-30, 32, 63, 67-68, 70 and 81-97 have been canceled.

Claims 3, 5-9 and 45-47 are currently withdrawn from consideration.

Claims 98-104 are added.

Claims 1, 2, 4, 10-14, 19-27, 31, 33-44, 48-62, 64-66, 69, 71-80, and 98-104 are currently under consideration.

Enablement rejections

The Office Action alleges that the specification is not enabling for a method to induce cardiomyogenesis by transfecting "*any kind of cell.*" Applicants disagree that transfection of cells or tissues other than cardiomyocytes or tissues that comprise cardiomyocytes is not enabled by the specification. Nevertheless, in an effort to expedite prosecution, the claims have been amended to recite that a polynucleotide of the invention is administered to a cardiomyocyte or tissue comprising cardiomyocytes. Applicants reserve the right to pursue claims directed to other types of cells or tissues during future prosecution. Note that the administration of a plasmid of the invention to cardiomyocytes or tissues comprising cardiomyocytes would also be expected to result in the

indications recited in the claims of Groups I, III and IV. See, *e.g.*, the disclosure in the specification that arteriogenesis, lymphangiogenesis, and vasculogenesis can be induced by the administration of the plasmid to the myocardium (at page 12, 4th full paragraph; page 13, 1st full paragraph; and page 12, 5th full paragraph, respectively).

The Office Action alleges that the specification is not enabling for a polynucleotide encoding "*any variant* of VEGF." Applicants disagree that the specification fails to enable the use of any of a variety of active variants of VEGF. Nevertheless, in an effort to expedite prosecution, the claims have been amended to recite that the VEGF is VEGF-165. Applicants reserve the right to pursue variants of VEGF during future prosecution. The claims recite VEGF-165 or an "active site" thereof. VEGF has been studied extensively, and a skilled worker can readily determine which sequences of the protein contain an active site. See, *e.g.*, the discussion at pages 21-22 of the specification. Therefore, the use of a polynucleotide encoding VEGF-165 or an active site thereof is fully supported by the specification.

The Office Action alleges that the specification is not enabling for the use of "*any viral or non-viral vector.*" Applicants maintain that the specification enables the use of any of a wide variety of vectors in methods of the invention. Nevertheless, in the interest of expediting prosecution, the claims have been amended to recite that the vector is a plasmid vector, in which inserted coding sequences are expressed under the control of a CMV promoter. Applicants reserve the right to pursue claims directed to other vectors during future prosecution.

With regard to the routes of administration that can be used in a method of the invention, any route that delivers a polynucleotide to cardiomyocytes or tissues comprising cardiomyocytes (the currently elected species) is enabled by the specification. Such routes include, *e.g.*, intravascular, intravenous, intra-arterial, intracardiac, intrapericardial, intramuscular, or a variety of other routes of administration. The Examiner has presented no evidence or sound scientific reasons to doubt the assertion of applicants that such modes of administration can be used successfully in a method of the invention. Arguments and references in support of some of these methods of administration - *e.g.*, intravascular administration - are discussed elsewhere in this Reply.

The Office Action quotes several references which allegedly indicate that gene transfer is unpredictable. Applicants wish to point out that the present specification clearly shows that a

method of the present claims can, *e.g.*, induce cardiomyogenesis in a large mammal (pig) model. Furthermore, a recent publication by the present inventors and colleagues (Vera Janavel *et al.* (2006) *Gene Ther.* 13, 1133-42), a copy of which is attached for the convenience of the Examiner, expands on the experiments described in Example VI of the specification. This publication confirms in a large animal model (sheep) that a method of the invention successfully induces mitosis of cardiomyocytes and a reduction of infarct size. Moreover, Vera Janavel *et al* (*supra*) clearly demonstrate the pattern and time course of gene expression at the molecular and histological levels (*i.e.*, mRNA and protein, *in vitro* and in myocardial tissue) after plasmid-mediated VEGF gene transfer. In view of this clear demonstration of efficacy and transfection efficiency, it is improper to reject the pending claims.

Rejection over Vale *et al.* (2000) *Circulation* 102, 965-974 ("Vale *et al.*")

Contrary to the allegation of the Office Action, Vale *et al.* neither anticipates nor renders obvious the instant claims.

At the outset, applicants wish to clarify that the terms "ischemia" and "infarction" are different and are not interchangeable. The attached pages from Stedman's Medical dictionary indicate that "ischemia," such as myocardial ischemia, is inadequate circulation of blood to a tissue (such as the myocardium), usually as a result of coronary artery disease. One form of ischemic tissue is sometimes referred to as "hibernating" tissue; this term is used in the Vale *et al.* reference. Ischemic tissue exhibits diminished function, but it is not necrotic (dead). By contrast, "infarct" or "infarction" is an area of necrosis [dead tissue] resulting from a sudden (and/or complete) insufficiency of arterial blood supply.

The Vale *et al.* reference reports that the administration of a plasmid encoding VEGF-165 can bring about an increase in myocardial function, as measured by the NOGA method; this increase is reported to be accompanied by augmented perfusion of the ischemic myocardial tissue. However, the reference does not teach or suggest that the dose of VEGF-165 which is administered therein is effective to induce cardiomyogenesis (mitosis or proliferation of the cells, which can, *e.g.*, lead to the replacement of dead cells in an infarcted area). In order to demonstrate this, Vale *et al* would have had to demonstrate an increased mitotic index, and/or a reduction in the size of infarcted

(dead) tissue. The induction of cardiomyogenesis is much more difficult to accomplish than merely restoring function to ischemic tissue; different dosing would be required to achieve these different effects.

The present claims recite a method by which, *e.g.*, the administration of a plasmid encoding VEGF-165 can stimulate mitosis of cardiomyocytes, and thereby, *e.g.*, achieve a reduction in infarct size. A summary of methods used to assay for mitosis is provided, *e.g.*, at pages 30-31 of the specification; and the results showing that mitosis and cell division do, in fact, occur, is provided, *e.g.*, at pages 32-33 and the figures and table referred to therein. Furthermore, Example VI, which is directed to the administration of a plasmid encoding VEGF-165 into sheep, is expanded upon in Vera Janavel *et al.*, (*supra*), which shows that administration of a plasmid encoding VEGF-165 (in a higher dose than used in the Vale *et al.* reference) results in stimulation of cardiomyocyte mitosis and reduction of infarct size.

As demonstrated in the Examples, the dose of VEGF used by the present inventors, which results in the mitosis of cardiomyocytes, is considerably (at least about 10- to 20-fold) higher than the dose used by Vale *et al.*, which is reported to reduce the size of defects in hibernating tissue. Vale *et al.* administered between about 250-500 μg total plasmid to human subjects (70 kg in weight), whereas the Examples in the present application report administering a total of 3.8 mg total to pigs (45 kg in weight). This higher dose resulted in the considerably more difficult achievement of inducing the cells to divide, not merely improving heart function. Therefore, the reference does not anticipate the instant claims, and provides no teaching or suggestion, with the requisite reasonable expectation of success, to use the higher dosages employed by the present inventors in order to induce cardiomyogenesis. Note that instant claims 59-62 recite doses of between about 0.008 and 0.36 (*e.g.*, 0.01 - 0.10) nmoles /kg; and new claims 98 and 99 further recite that these dosages are greater than about 0.04 mg/kg (which is equivalent to about 0.0165 nmole/ kg). These dosages reflect the dosages used in the present Examples, rather than the much lower dosages used in Vale *et al.*

Furthermore, the dose of VEGF which is sufficient to induce cardiomyogenesis is administered at a much higher concentration (*e.g.*, about 1.9 mg/ml in Example III) than the concentration used by Vale *et al.* (0.03-0.06 mg/ml). Without wishing to be bound by any particular

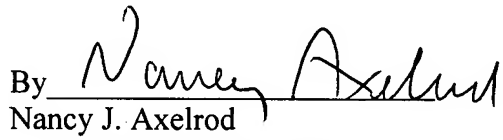
mechanism, it is suggested that this higher concentration contributes to an increase in transfection efficiency and/or entry of the DNA into the nucleus. Note that claim 64 recites that the concentration of a plasmid administered to a subject is between about 0.5 and about 4 mg/ml.

In view of the preceding arguments and amendments, it is believed that the application is in condition for allowance, which action is respectfully requested.

The Commissioner is hereby authorized to charge any fees association with this response or credit any overpayment to Deposit Account No. 22-0261, citing Docket No. 31978-201641.

Dated: June 29, 2007

Respectfully submitted,

By 

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STEDMAN'S Medical Dictionary

27th Edition

Illustrated in Color

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eddish brown, lustrous granules, odorless or nearly so; used in inemia.

. **alum,** SYN *ferric* ammonium sulfate.

. **filings,** small packets of *Paragonimus* spp. eggs that can be een in the sputum; the egg clumps tend to be yellow-brown.

. **protoporphyrin,** a protoporphyrin to which an i. atom is com-lexed; e.g., heme.

. **pyri'tes,** native sulfide of i.

on-52 (^{52}Fe). A radioactive iron isotope; a cyclotron-produced ositron emitter with a half-life of 8.28 h, used to study iron netabolism.

on-55 (^{55}Fe). An iron isotope; a positron emitter with a half-ife of 2.73 years; used (less often than ^{59}Fe) as a tracer in study of ron metabolism and in blood perfusion studies.

on-59 (^{59}Fe). An iron isotope; a gamma and beta emitter with a alf-life of 44.51 days; used as tracer in the study of iron metabo-ism, determination of blood volume, and in blood transfusion tudies.

ra·di·ate (i-rā'dē-āt). To apply radiation from a source to a tructure or organism. [see irradiation]

ra·di·a·tion (i-rā-dē-ā'shŭn). **1.** The subjective enlargement of bright object seen against a dark background. **2.** Exposure to the ction of electromagnetic radiation (e.g., heat, light, x-rays). **3.** he spreading of nervous impulses from one area in the brain or ord, or from a tract, to another tract. SEE ALSO radiation. [L. *ir-adio, (in-r)*, pp. *-radi-atus,* to beam forth]

ra·tion·al (i-rash'ŭn-ăl). Not rational; unreasonable (contrary o reason) or unreasoning (not exercising reason). [L. *irrationalis,* vithout reason]

re·duc·i·ble (ir-rē-doo'si-bl, i-rē-). **1.** Not reducible; incapable f being made smaller. **2.** In chemistry, incapable of being made impler, or of being replaced, hydrogenated, or reduced in posi-ve charge.

re·spir·a·ble (ir-rē-spīr'ă-bl). **1.** Incapable of being inhaled ecause of irritation to the airway, resulting in breath-holding. **2.** Denoting a gas or vapor either poisonous or containing insuffi-ient oxygen. **3.** Denoting an aerosol composed of particles with erodynamic size larger than 10 μ.

re·spon·si·bil·i·ty (ir'rē-spons-i-bil'i-tē). The state of not act-ng in a manner that is responsible, for conscious or unconscious easons.

riminal i., the state, usually attributed to mental defect or isease, that renders a person not responsible for criminal con-uct.

re·sus·ci·ta·ble (ir'rē-sŭs'i-tă-bl). Incapable of being revived.

re·vers·i·ble (ir-rē-ver'si-bl). Incapable of being reversed; per-anent. [L. *in-* (*ir-*) neg. + *re-verto,* pp. *-versus,* to turn back]

ri·gate (ir'i-gāt). To perform irrigation. [L. *ir-rigo,* pp. *-atus,* to rigate, fr. *in,* on, + *rigo,* to water]

ri·ga·tion (ir-i-gā'shŭn). The washing out of a body cavity, pace, or wound with a fluid. [see irrigate]

ri·ga·tor (ir'i-gā-ter). An appliance used in irrigation.

ri·ta·bil·i·ty (ir'i-tă-bil'i-tē). The property inherent in proto-lasm of reacting to a stimulus. [L. *irritabilitas,* fr. *irrito,* pp. *itus,* to excite]

lectric i., the response of a nerve or muscle to the passage of a urrent of electricity; in cases of degeneration in nerve or muscle his i. is altered or lost. SEE modal *alteration,* qualitative *altera-on,* quantitative *alteration.*

yotatic i., the ability of a muscle to contract in response to the timulus produced by a sudden stretching.

ri·ta·ble (ir'i-tă-bl). **1.** Capable of reacting to a stimulus. **2.** 'ending to react immoderately to a stimulus. Cf. excitable.

ri·tant (ir'i-tant). **1.** Irritating; causing irritation. **2.** Any agent vith this action.

rimary i., a substance that causes inflammation and other evi-ence of irritation, particularly of the skin, on first contact or xposure, or as a reaction to cumulative contacts, not dependent [illegible]

or muscle to a stimulus. **3.** The evocation of a normal or exagger ated reaction in the tissues by the application of a stimulus. [L. *irritatio*]

ir·ri·ta·tive (ir-i-tā'tiv). Causing irritation.

ir·ru·ma·tion (ir'oo-mā'shŭn). SYN fellatio. [L. *irrumo,* pp. *-atus,* to give suck]

ir·rup·tion (i-rŭp'shŭn). Act or process of breaking through to a surface. [L. *irruptio,* fr. *irrumpo,* to break in]

ir·rup·tive (i-rŭp'tiv). Relating to or characterized by irruption.

IRS-1 Abbreviation for insulin receptor *substrate-1*.

IRV Abbreviation for inspiratory reserve *volume*.

Irvine, A. Ray, Jr., U.S. ophthalmologist, *1917. SEE I.-Gass *syndrome*.

ISA Abbreviation for intrinsic sympathomimetic *activity*.

Is·a·mine blue (is'ă-mēn, ī'să-). SYN pyrrol blue.

is·aux·e·sis (ī-sawk-zē'sis). Growth of parts at the same rate as growth of the whole. [G. *isos,* even, + *auxēsis,* increase]

is·che·mia (is-kē'mē-ă). Local anemia due to mechanical obstruc-tion (mainly arterial narrowing or disruption) of the blood supply. [G. *ischō,* to keep back, + *haima,* blood]

myocardial i., inadequate circulation of blood to the myocardi-um, usually as a result of coronary artery disease. SEE ALSO *angina* pectoris, myocardial *infarction*.

postural i., the reduced blood pressure and flow induced in a part, e.g., the leg or foot, by raising it above the heart level; used to reduce bleeding during surgical operations on the extremities.

i. ret'inae, diminished blood supply in the retina due to failure of the arterial circulation; it may occur as a result of arterial embo-lism or spasm; poisoning, as by quinine; or exsanguination from recurring profuse hemorrhages; bilateral transitory or permanent blindness may result.

silent i., myocardial i. without accompanying signs or symptoms of angina pectoris; can be detected by ECG and other lab tech-niques. SEE ALSO silent myocardial *infarction*.

is·che·mic (is-kē'mik). Relating to or affected by ischemia.

is·che·sis (is-kē'sis). Suppression of any discharge, especially of a normal one. [G. *ischō,* to hold back]

is·chia (is'kē-ă). Plural of ischium.

is·chi·ad·ic (is-kē-ad'ik). SYN sciatic (1).

is·chi·a·di·cus (is-kē-ad'i-kŭs). SYN sciatic. [L.]

is·chi·al (is'kē-ăl). SYN sciatic (1).

is·chi·al·gia (is-kē-al'jē-ă). **1.** Obsolete term for pain in the hip; specifically, the ischium. SYN ischiodynia. **2.** Obsolete term for sciatica. [G. *ischion,* hip, + *algos,* pain]

is·chi·at·ic (is-kē-at'ik). SYN sciatic (1).

ischio-. The ischium. [G. *ischion,* hip joint, haunch (ischium)]

is·chi·o·a·nal (is-kē-ō-ā'năl). Relating to the ischium and the anus.

is·chi·o·bul·bar (is-kē-ō-bŭl'bar). Relating to the ischium and the bulb of the penis.

is·chi·o·cap·su·lar (is-kē-ō-kap'soo-lăr). Relating to the ischium and the capsule of the hip joint; denoting that part of the capsule which is attached to the ischium.

is·chi·o·cav·er·no·sus. SEE ischiocavernous (*muscle*).

is·chi·o·cav·ern·ous (is-kē-ō-kav'er-nŭs). Relating to the ischi-um and the corpus cavernosum.

is·chi·o·cele (is'kē-ō-sēl). SYN sciatic *hernia*. [ischio- + G. *kēlē,* hernia]

is·chi·o·coc·cyg·e·al (is-kē-ō-kok-sij'ē-ăl). Relating to the ischi-um and the coccyx.

is·chi·o·coc·cyg·e·us (is-kē-ō-kok-sij'ē-ŭs). SYN coccygeus *mus-cle*. SEE muscle.

is·chi·o·dyn·ia (is'kē-ō-din'ē-ă). SYN ischialgia (1). [ischio- + G. *odynē,* pain]

is·chi·o·fem·o·ral (is-kē-ō-fem'ŏ-răl). Relating to the ischium, or hip bone, and the femur, or thigh bone.

is·chi·o·fib·u·lar [illegible]

suspected arthropod vector; proglottids, eggs, and egg capsules resemble those of *Raillietina* spp.

in·ert (in-ert'). **1.** Slow in action; sluggish; inactive. **2.** Devoid of active chemical properties, as the inert gases. **3.** Denoting a drug or agent having no pharmacologic or therapeutic action. [L. *iners*, unskillful, sluggish, fr. *in*, neg. + *ars*, art]

in·er·tia (in-er'shē-ă, in-er'shăh). **1.** The tendency of a physical body to oppose any force tending to move it from a position of rest or to change its uniform motion. **2.** Denoting inactivity or lack of force, lack of mental or physical vigor, or sluggishness of thought or action. [L. want of skill, laziness]

magnetic i., SYN hysteresis (2).

psychic i., a psychiatric term denoting resistance to any change in ideas or to progress; fixation of an idea.

uterine i., absence of effective uterine contractions during labor; **primary uterine i., true uterine i.,** uterine i. that occurs when the uterus fails to contract with sufficient force to effect continuous dilation or effacement of the cervix or descent or rotation of the fetal head, and when the uterus is easily indentable at the acme of contraction; **secondary uterine i.,** uterine i. that occurs when the uterine contractions are initially vigorous but then decrease in vigor, and the progress of labor ceases.

in ex·tre·mis (in eks-trē'mis). At the point of death. [L. *extremus*, last]

in·fan·cy (in'fan-sē). Babyhood; the earliest period of extrauterine life; roughly, the first year of life.

in·fant. A child under the age of 1 year. [L. *infans*, not speaking]

i. Hercules, term applied to young children with precocious sexual and muscular development due to a virilizing adrenocortical disorder.

liveborn i., the product of a livebirth; an i. who shows evidence of life after birth; life is considered to be present after birth if any one of the following is observed: 1) if the infant breathes; 2) if the infant shows beating of the heart; 3) if pulsation of the umbilical cord occurs; or 4) if there is definite movement of voluntary muscles.

postmature i., a baby born after over 42 weeks of gestation, which puts the child at risk because of inadequate placental function. The infant usually shows wrinkled skin, sometimes more serious abnormalities.

postterm i., an i. with a gestational age of 42 completed weeks or more (294 days or more).

preterm i., an i. with gestational age of more than 20 weeks and less than 37 completed weeks (259 completed days).

stillborn i., an i. who has achieved 20 weeks of gestation and shows no evidence of life after birth. Cf. liveborn i.

term i., an i. with gestational age between 37 completed weeks (259 completed days) and 42 completed weeks (294 completed days).

in·fan·ti·cide (in-fan'ti-sīd). **1.** The killing of an infant. **2.** One who murders an infant. [infant + L. *caedo*, to kill]

in·fan·tile (in'făn-tīl). **1.** Relating to, or characteristic of, infants or infancy. **2.** Denoting childish behavior.

in·fan·ti·lism (in-fan'ti-lizm). **1.** A state marked by slow development of mind and body. SYN infantile dwarfism. **2.** Childishness, as characterized by a temper tantrum of an adolescent or adult. **3.** Underdevelopment of the sexual organs.

Brissaud i., SYN infantile *hypothyroidism*.

dysthyroidal i., SYN infantile *hypothyroidism*.

hepatic i., delayed development as a result of liver disease.

hypophysial i., growth hormone deficiency due to failure of hypothalamic growth hormone-releasing hormone (also known as somatocrinin.)

hypothyroid i., SYN infantile *hypothyroidism*.

idiopathic i., dwarfism generally associated with hypogonadism; may be caused by deficient secretion of anterior pituitary hormones. SYN Lorain disease, proportionate i., universal i.

Lorain-Lévi i., SYN pituitary *dwarfism*.

myxedematous i., SYN infantile *hypothyroidism*.

pancreatic i., i. associated with deficiency or absence of pancreatic secretion.

pituitary i., SYN pituitary *dwarfism*.

proportionate i., SYN idiopathic i.

renal i., SYN renal *rickets*.

sexual i., failure to develop secondary sexual characteristics after the normal time of puberty.

static i., a condition observed in young children resembling spastic spinal paralysis; it is marked by hypotonia of the muscles of the trunk and hypertonia of the muscles of the extremities.

tubal i., a term descriptive of a corkscrew-like fallopian tube as seen in fetal life.

universal i., SYN idiopathic i.

in·farct (in'farkt). An area of necrosis resulting from a sudden insufficiency of arterial or venous blood supply. SYN infarction (2). [L. *in-farcio*, pp. *-fartus* (*-ctus*, an incorrect form), to stuff into]

anemic i., an i. in which little or no bleeding into tissue spaces occurs when the blood supply is obstructed. SYN pale i., white i. (1).

bland i., an uninfected i.

bone i., an area of bone tissue that has become necrotic as a result of loss of its arterial blood supply.

Brewer i.'s, dark-red, wedge-shaped areas resembling i.'s, seen on section of a kidney in pyelonephritis.

embolic i., an i. caused by an embolus.

hemorrhagic i., an i. red in color from infiltration of blood from collateral vessels into the necrotic area. SYN hemorrhagic gangrene (1), red i.

pale i., SYN anemic i.

red i., SYN hemorrhagic i.

Roesler-Dressler i., myocardial infarction in dumbbell form involving the anterior and posterior left ventricle and the left side of the ventricular septum.

septic i., an area of necrosis resulting from vascular obstruction by emboli composed of clumps of bacteria or infected material.

thrombotic i., an i. caused by a thrombus.

uric acid i., precipitates of uric acid distending renal collecting tubules in the newborn; since there is no necrosis, the term infarct is a misnomer.

white i., (1) SYN anemic i; (2) in the placenta, intervillous fibrin with ischemic necrosis of villi.

Zahn i., a pseudoinfarct of the liver, consisting of an area of congestion with parenchymal atrophy but no necrosis; due to obstruction of a branch of the portal vein.

Zahn infarct: liver section

in·farc·tion (in-fark'shŭn). **1.** Sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, mechanical factors or pressure that produces a macroscopic area of necrosis; any organ can be affected. **2.** SYN infarct.

anterior myocardial i., i. involving the anterior wall of the left ventricle, and producing indicative electrocardiographic changes in the anterior chest leads and often in limb leads, I and aVL.

anteroinferior myocardial i., i. involving both anterior and inferior walls of the heart simultaneously.

anterolateral myocardial i., extensive anterior i. producing indicative changes across the precordium, often also on leads I and aVL.

anteroseptal myocardial i., an anterior i. in which indicative electrocardiographic changes are confined to the medial chest leads (V_1–V_4).

apical i., SYN inferolateral myocardial i.

cardiac i., SYN myocardial i.

diaphragmatic myocardial i., SYN inferior myocardial i.

Freiberg i., SYN Freiberg *disease*.

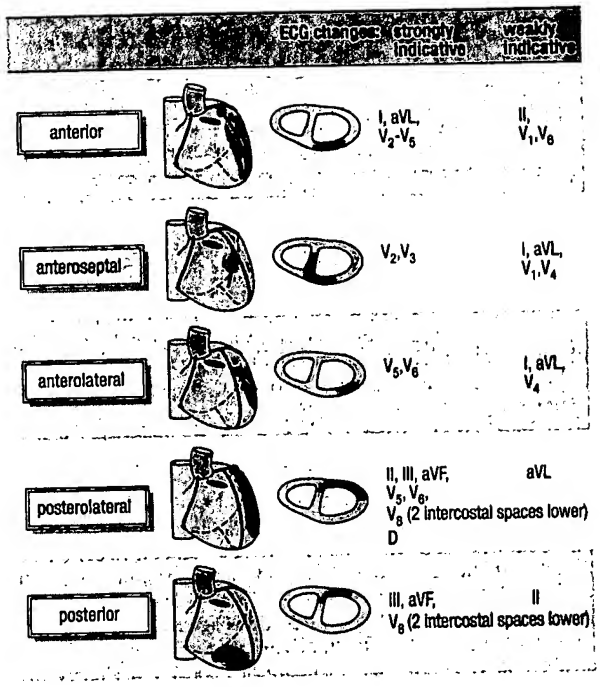
inferior myocardial i., i. in which the inferior or diaphragmatic wall of the heart is involved, producing indicative changes in leads II, III, and aVF in the electrocardiogram. SYN diaphragmatic myocardial i.

inferolateral myocardial i., i. involving the inferior and lateral surfaces of the heart and producing indicative changes in the electrocardiogram in leads II, III, aVF, V_5, and V_6. SYN apical i.

lateral myocardial i., i. involving only the lateral wall of the heart, producing indicative electrocardiographic changes confined to leads I, aVL, or V_5 and V_6.

myocardial i. (MI), i. of an segment of the heart muscle, usually as a result of occlusion of a coronary artery. SYN cardiac i., heart attack.

Myocardial infarction is the most common cause of death in the U.S. About 800,000 people annually sustain first heart attacks, with a mortality rate of 30%, and 450,000 people sustain recurrent heart attacks, with a mortality rate of 50%. The most common cause of MI is thrombosis of an atherosclerotic coronary artery. Less common causes are coronary artery anomalies, vasculitis, or spasm induced by cocaine, ergot derivatives, or other agents. Risk factors for MI include male gender, family history of MI, obesity, hypertension, cigarette smoking, and elevation of total cholesterol, LDL cholesterol, homocysteine, lipoprotein (a), or C-reactive protein. At least 80% of MIs occur in people without a prior history of angina pectoris, and 20% are not recognized, either because they cause no symptoms (silent infarction) or because symptoms are attributed to other causes. Some 20% of people sustaining MI die before reaching a hospital. The classical symptom of MI is crushing anterior chest pain radiating into the neck, shoulder, or arm, lasting more than 30 minutes, and not relieved by nitroglycerin; typically pain is accompanied by dyspnea, diaphoresis, weakness, and nausea. Significant physical findings, often absent, include an atrial gallop rhythm (4th heart sound) and a pericardial friction rub. The electrocardiogram shows ST segment elevation (later changing to depression) and T wave inversion in leads reflecting the area of infarction. Q waves indicate transmural damage and a poorer prognosis. Diagnosis is supported by acute elevation in serum levels of CK-MB, lactic dehydrogenase, the myoglobin isoenzyme of creatine kinase, and troponins. Unequivocal evidence of MI may be lacking during the first 6 hours in as many as 50% of patients. Death from acute MI is usually due to arrhythmia (ventricular fibrillation or asystole), shock (forward failure), congestive heart failure, or papillary muscle rupture. Other grave complications, which may occur during convalescence, include cardiorrhexis, ventricular aneurysm, and mural thrombus. Acute MI is treated (ideally under continuous ECG monitoring in the intensive care or coronary care unit of a hospital) with narcotic analgesics, oxygen by inhalation, intravenous administration of a thrombolytic agent, antiarrhythmic agents when indicated, and usually anticoagulants (aspirin, heparin), beta-blockers, and ACE inhibitors. Patients with evidence of persistent ischemia require angiography and may be candidates for balloon angioplasty. Data from the Framingham Heart Study show that a higher percentage of acute MIs are silent or unrecognized in women and the elderly. Several studies have shown that women and the elderly tend to wait longer before seeking medical care after the onset of acute coronary symptoms than men and younger persons. In addition, women seeking emergency treatment for symptoms suggestive of acute coronary disease are less likely than men with similar symptoms to be admitted for evaluation, and women are less frequently referred than are men for diagnostic tests such as coronary angiography. Other studies have shown important gender differences in the presenting symptoms and medical recognition of MI. Chest pain is the most common symptom reported by both men and women, but men are more likely to complain of diaphoresis, while women are more likely to experience neck, jaw, or back pain, nausea, vomiting, dyspnea, or cardiac failure, in addition to chest pain. The incidence rates of acute pulmonary edema and cardiogenic shock in MI are higher in women, and mortality rates at 28 days and 6 months are also higher.



myocardial infarction

nontransmural myocardial i. (NTMI), necrosis of heart muscl that fails to extend from the endocardium completely to the ep cardium, often erroneously considered relatively benign.

posterior myocardial i., i. involving the posterior wall of th heart; also formerly used erroneously of i.'s involving the inferi or diaphragmatic surface of the heart.

silent myocardial i., i. that produces none of the characterist symptoms and signs of myocardial i.

subendocardial myocardial i., i. that involves only the layer muscle subjacent to the endocardium.

through-and-through myocardial i., SYN transmural myocardi i.

transmural myocardial i., i. that involves the whole thickness the heart muscle from endocardium to epicardium. SYN throug and-through myocardial i.

watershed i., cortical i. in an area where the distribution of maj cerebral arteries meet or overlap.

in·fect (in-fekt'). **1.** For a microorganism to enter, invade, inhabit another organism, causing infection or contamination. To dwell internally, endoparasitically, as opposed to external (infest). [L. *in-ficio,* pp. *-fectus,* to dip into, dye, corrupt, infect, *in* + *facio,* to make]

in·fec·tion (in-fek'shŭn). Invasion of the body with organis that have the potential to cause disease.

agonal i., SYN terminal i.

Section 1

NAKED DNA, OLIGONUCLEOTIDE AND PHYSICAL METHODS

1

The Mechanism of Naked DNA Uptake and Expression

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ABSTRACT

The administration of naked nucleic acids into animals is increasingly being used as a research tool to elucidate mechanisms of gene expression and the role of genes and their cognate proteins in the pathogenesis of disease in animal models (Herweijer and Wolff, 2003; Hodges and Scheule, 2003). It is also being used in several human clinical trials for genetic vaccines, Duchenne muscular dystrophy, peripheral limb ischemia, and cardiac ischemia (Davis *et al.*, 1996; Romero *et al.*, 2002; Tsurumi *et al.*, 1997). Naked DNA is an attractive non-viral vector because of its inherent simplicity and because it can easily be produced in bacteria and manipulated using standard recombinant DNA

Advances in Genetics, Vol. 54
0065-2660/05 $35.00

DOI: 10.1016/S0065-2660(05)54001-X

techniques. It shows very little dissemination and transfection at distant sites following delivery and can be readministered multiple times into mammals (including primates) without inducing an antibody response against itself (i.e., no anti-DNA antibodies generated) (Jiao *et al.*, 1992). Also, contrary to common belief, long-term foreign gene expression from naked plasmid DNA (pDNA) is possible even without chromosome integration if the target cell is postmitotic (as in muscle) or slowly mitotic (as in hepatocytes) and if an immune reaction against the foreign protein is not generated (Herweijer *et al.*, 2001; Miao *et al.*, 2000; Wolff *et al.*, 1992; Zhang *et al.*, 2004). With the advent of intravascular and electroporation techniques, its major restriction—poor expression levels—is no longer limiting and levels of foreign gene expression *in vivo* are approaching what can be achieved with viral vectors.

Direct *in vivo* gene transfer with naked DNA was first demonstrated when efficient transfection of myofibers was observed following injection of mRNA or pDNA into skeletal muscle (Wolff *et al.*, 1990). It was an unanticipated finding in that the use of naked nucleic acids was the control for experiments designed to assess the ability of cationic lipids to mediate expression *in vivo*. Subsequent studies also found foreign gene expression after direct injection in other tissues such as heart, thyroid, skin, and liver (Acsadi *et al.*, 1991; Hengge *et al.*, 1996; Kitsis and Leinwand, 1992; Li *et al.*, 1997; Sikes and O'Malley 1994; Yang and Huang, 1996). However, the efficiency of gene transfer into skeletal muscle and these other tissues by direct injection is relatively low and variable, especially in larger animals such as nonhuman primates (Jiao *et al.*, 1992).

After our laboratory had developed novel transfection complexes of pDNA and amphipathic compounds and proteins, we sought to deliver them to hepatocytes *in vivo* via an intravascular route into the portal vein. Our control for these experiments was naked pDNA and we were once again surprised that this control group had the highest expression levels (Budker *et al.*, 1996; Zhang *et al.*, 1997). High levels of expression were achieved by the rapid injection of naked pDNA in relatively large volumes via the portal vein, the hepatic vein, and the bile duct in mice and rats. The procedure also proved effective in larger animals such as dogs and nonhuman primates (Eastman *et al.*, 2002; Zhang *et al.*, 1997). The next major advance was the demonstration that high levels of expression could also be achieved in hepatocytes in mice by the rapid injection of naked DNA in large volumes simply into the tail vein (Liu *et al.*, 1999; Zhang *et al.*, 1999). This hydrodynamic tail vein (HTV) procedure is proving to be a very useful research tool not only for gene expression studies, but also more recently for the delivery of small interfering RNA (siRNA) (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002).

The intravascular delivery of naked pDNA to muscle cells is also attractive particularly since many muscle groups would have to be targeted for intrinsic muscle disorders such as Duchenne muscular dystrophy. High levels of gene expression were first achieved by the rapid injection of naked DNA in large volumes via an artery route with both blood inflow and outflow blocked surgically (Budker *et al.*, 1998; Zhang *et al.*, 2001). Intravenous routes have also been shown to be effective (Hagstrom et al., 2004; Liang *et al.*, 2004; Liu *et al.*, 2001). For limb muscles, the ability to use a peripheral limb vein for injection and a proximal, external tourniquet to block blood flow renders the procedure to be clinically viable.

This review concerns itself with the mechanism by which naked DNA is taken up by cells *in vivo*. A greater understanding of the mechanisms involved in the uptake and expression of naked DNA, and thus connections between postulated mechanisms and expression levels, is emphasized. Inquiries into the mechanism not only aid these practical efforts, but are also interesting on their own account with relevance to viral transduction and cellular processes. The delivery to hepatocytes is first discussed given the greater information available for this process, and then uptake by myofibers is discussed.

I. OVERVIEW OF HEPATOCYTE DELIVERY

As with studies involving viral transduction, the delivery of naked nucleic acids can be broken down into several transport steps, including (1) transport of nucleic acids from the site of injection to the liver, (2) exit of nucleic acids out of the liver vessel lumen into contact with hepatocytes, (3) uptake by hepatocytes, (4) entry into the cytoplasm, and (5) entry into the nucleus.

The key destination for the journey of typical plasmid DNA expression vectors is the nucleus where the plasmids utilize RNA polymerase II promoters to transcribe messenger RNA for expression of the desired foreign gene. An important observation that has to be incorporated into any mechanism hypothesis is that foreign gene expression from a variety of promoters has only been observed in hepatocytes. This suggests that the plasmid DNA only enters the nucleus of hepatocytes and not any of the nonparenchymal cells, such as endothelial cells or Kupffer cells that line the sinusoidal space. For siRNA, only cytoplasmic entry is required to access the RISC complex and RNA interference effects have been best documented in hepatocytes.

Except for actual liver delivery, studies involving the direct injection of nucleic acids into liver vessels (portal vein, hepatic vein, and bile duct) are considered together with studies using tail vein injections because it is assumed that the basic mechanism is the same for the two different routes of administration.

II. TRANSPORT OF NUCLEIC ACIDS FROM INJECTION SITE TO LIVER

For tail vein injections, this point requires some discussion but the process of injecting nucleic acid directly into liver vessels obviously avoids this delivery hurdle. Direct observation of the liver shows that the liver swells during the tail vein injection procedure (Zhang *et al.*, 1999, 2004) (Fig. 1.1). Efficient gene expression and delivery of the plasmid DNA to the liver both require that the DNA be injected rapidly and in a large volume. Similarly, siRNA or morpholinooligonucleotide suppression of gene expression also requires that the nucleic acid be injected into the tail vein using the HTV procedure. For example, fluorescently labeled pDNA or siRNA was only associated with the liver when injected using the HTV procedure but not after normal tail vein (NTV) injections using low volumes and injection speeds.

Southern blot and polymerase chain reaction (PCR) analysis provided quantitative information concerning the effect of the HTV procedure on liver

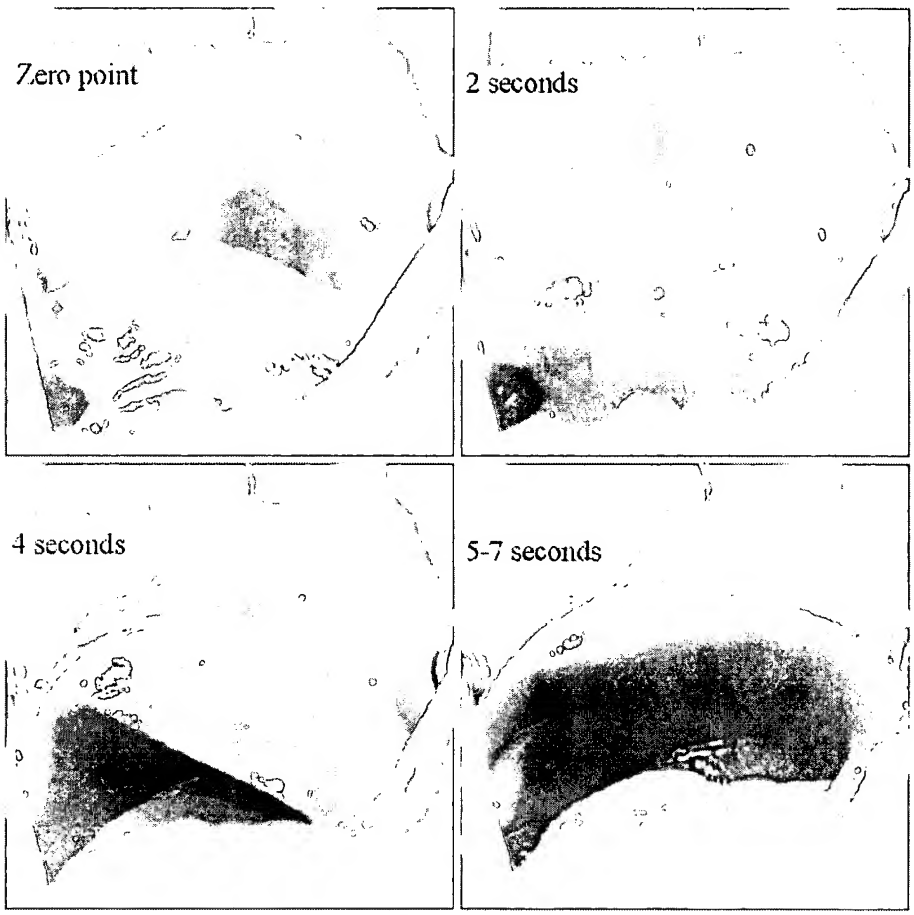

Figure 1.1. Liver at various times after HTV injection. The liver immediately swells and becomes pale, indicating that it fills with the injection fluid.

delivery (Kobayahi *et al.*, 2001). Immediately after HTV injection of radioactive DNA, 50% of the radioactive signal was associated with the liver and 20% remained in the plasma, whereas after NTV injection, ~30% of the radioactive DNA was associated with the liver and ~55% was in the plasma. As explained later, the high baseline liver uptake of 30% after NTV is probably due to Kupffer and endothelial cell uptake.

The most likely mechanism by which the HTV procedure enables efficient liver delivery is that rapid injection of the large volume causes a transient right-sided CHF that leads to back pressure of the nucleic solution retrograde from the IVC to the hepatic vein and back up into the liver. Consistent with this postulated mechanism is that there is a transient decrease in heart rate and an increase in IVC pressure immediately after injection (Zhang *et al.*, 2004).

III. EXTRAVASTION OF INJECTED NUCLEIC ACID

Given that plasmid DNA has a gyration ratio of 0.1 μm and that sinusoid fenestrae have an average diameter also of ~0.1 μm, simple diffusion of the pDNA would not enable sufficient pDNA extravasation for expression. If fluorescently labeled pDNA is injected into the portal vein without substantially increasing the intravascular pressure, then little pDNA is observed out of the sinusoids and is associated with parenchymal cells. However, if injected under high-pressure conditions, then much more labeled pDNA becomes associated with hepatocytes (Budker *et al.*, 2000). Similarly, much more fluorescently labeled pDNA becomes associated with hepatocytes if the pDNA is injected under HTV conditions. For greater quantitative assessment, twofold more radioactively labeled DNA becomes associated with hepatocytes if injected under HTV conditions (Lecocq *et al.*, 2003).

For injections into liver vessels, blocking the outflow of the injected fluid enables the intravascular pressure to rise and extravasation of pDNA. The ability of the HTV procedure to extravasate pDNA may be related to the tortuousness of the hepatic veins coming into the sinusoids (Zhang *et al.*, 2004). This anatomical feature may provide increased resistance to outflow, thereby enabling an increased intravascular pressure. Thus, increased intravascular pressure within the liver, an effect common to both direct liver blood vessel and HTV injections, appears to be a prerequisite for pDNA delivery to hepatocytes.

The resulting increased intravascular pressure has been postulated to enhance the extravasation of pDNA by enlarging the sinusoidal fenestrae. Consistent with this hypothesis are scanning electron micrograph (EM) images that show enlarged fenestrae after HTV injection (Zhang *et al.*, 2004). In

addition, we have found large amounts of DNA in the space of Disse after HTV injection (Fig. 1.2A). Convective flow of pDNA within the injection fluid out of the intravascular space would also be required to account for the increased hepatocyte delivery. Viral vectors such as lentiviral vectors also have had more expression when delivered in increased volume in mice (Condiotti *et al.*, 2004).

After portal vein, hepatic vein, or HTV injection, not all of the hepatocytes do not take up the injected nucleic acid. Depending on the exact injection conditions and the type of nucleic acid (pDNA or siRNA), somewhere between 5 and 70% of the hepatocytes can be seen to take up the labeled nucleic acid (Rossmanith *et al.*, 2002). This is consistent with the general experience that less than 50% of hepatocytes can be made to express a foreign gene or be inhibited by siRNA. The restriction of nucleic acid delivery to a subset of hepatocytes may be related to limited pDNA extravasation in certain areas of the liver. For example, pDNA expression is greater around the central vein after HTV and hepatic vein injections. Interestingly, we have not found expression greater around the portal vein after portal vein injections.

IV. CYTOPLASMIC ENTRY

After HTV injection, pDNA could enter the cytoplasm via endocytic vesicles or transient membrane pores; evidence in support and against each of these hypotheses is discussed.

While the initial phenomenon was first described using pDNA, a variety of molecules can be taken up after HTV injection, including PCR fragments (Hofman *et al.*, 2001). Concerning nucleic acids and its analogues, gene expression has been blocked using either siRNA or morpholino antisense molecules (Lewis *et al.*, 2002; McCaffrey *et al.*, 2002, 2003). Fluorescently labeled siRNA is observed in up to 60% of hepatocytes, mostly in their nuclei. Proteins such as *Escherichia coli* ß-galactosidase, IgG, and bovine serum albumin can also enter hepatocytes after HTV (Kobayahi *et al.*, 2001; Zhang *et al.*, 2004). In addition, small molecules such as Evans blue can also be taken up (Zhang *et al.*, 2004). The ability of a variety of molecules to be taken up by hepatocytes after HTV injection suggests that the uptake process is not receptor mediated. However, the HTV procedure did not increase liver uptake of radioactive PEG (4000 molecular weight), suggesting that some molecules may not enter or are not retained by hepatocytes (Kobayahi *et al.*, 2001).

The possibility of a receptor-mediated uptake process was raised on the basis of competition experiments. Luciferase expression was inhibited when a luciferase pDNA vector was coinjected with a variety of polyanions into the portal vein (under high pressure conditions) or by HTV injection (Budker *et al.*, 2000). A putative, 75- to 80-kDa receptor for oligonucleotide uptake has been

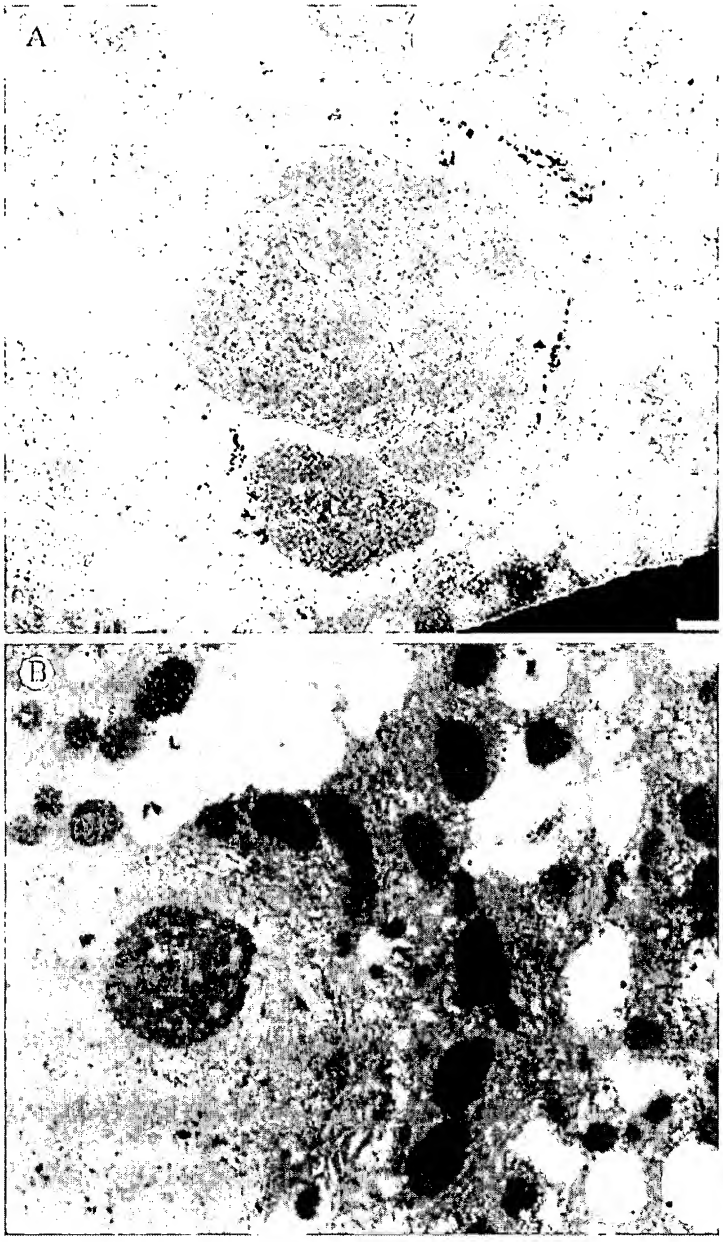

Figure 1.2. Electron micrographs showing pDNA distribution in liver after HTV injection. DNP-modified pDNA was injected in ICR mouse by HTV and 5 min after injection liver was isolated and fixed with formaldehyde/glutaraldehyde. EM sections were stained with anti-DNP antibodies and gold-labeled secondary antibodies. DNA can be seen as small black spots. (A) DNA in space of Disse and large hepatic vesicles. (B) DNA in hepatic nuclei and vesicles.

noted to be present on hepatocytes (Vlassov *et al.*, 1994). Another study found that a variety of polyanions, dextran sulfate, heparin, poly I. and poly C failed to inhibit the uptake of radioactive DNA by the liver nor luciferase expression (Kobayahi *et al.*, 2001). However, this study did not coinject these excess polyanions with the DNA under high pressure but preinjected them under NTV conditions. Nonetheless, we have repeated our competition experiments using HTV injections and found that polyanions had a less inhibitory effect than previously noted, but excess nonexpressing DNA still consistently inhibited luciferase expression. It has also been found that reporter gene expression is a saturating process, which is consistent with a receptor-mediated process (Rossmanith *et al.*, 2002). While we took care to exclude the possibility that excess DNA and polyanions decreased pDNA uptake or expression by inhibiting transport steps other than cellular uptake (e.g., nuclear entry), such postuptake effects may still be operative.

Hepatocyte membrane pores after HTV injection have been observed using scanning EM (Zhang *et al.*, 2004). The quick onset of elevated ALT and AST blood levels 2 h after injection is also consistent with the appearance of membrane pores (Rossmanith *et al.*, 2002). It has also been proposed that increased expression is generated via pores that are induced either by massaging the liver or by repetitive injections (Hickman *et al.*, 1995; Liu and Huang, 2002; Liu *et al.*, 2004).

EM studies also show that the HTV procedure causes an increased number of vesicles to appear in hepatocytes (V. Budker *et al.*, manuscript in preparation). In fact, gold-labeled pDNA can be seen in these vesicles (Fig. 1.2). Subcellular fractionation studies (done by centrifugation) indicated that radioactively labeled DNA after HTV injection becomes associated with vesicles associated with hepatocytes (Lecocq *et al.*, 2003). Curiously, the DNA is sensitive to DNase degradation (Lecocq *et al.*, 2003). It has been proposed that adenovirus entry into cells occurs via macropinosomes that are induced by the adenovirus interaction with specific cellular receptors and that are intrinsically leaky (Meier *et al.*, 2002). Perhaps pDNA entry after HTV injection occurs by a similar process in which the HTV procedure induces vesicles that take up pDNA and from which the pDNA leaks out.

One interesting observation is that if a mouse is first injected with only fluid using the HTV procedure and is then immediately injected with pDNA using the NTV, substantial luciferase expression can still be observed (Andrianaivo *et al.*, 2004; Zhang *et al.*, 2004). In fact, the NTV injection of the expression pDNA vector can be delayed for several minutes with expression still possible, albeit at lower levels than if injected just using the HTV procedure. For example, if the expressing pDNA is injected 12 or 45 s after HTV injection, expression falls to 30- or 10,000-fold, respectively, lower than the value achieved when the pDNA is injected by the HTV procedure

(Andrianaivo *et al.*, 2004). Another study found that a 30-s delay causes a ~20-fold drop in expression (Zhang *et al.*, 2004). Furthermore, Evans blue uptake can still be taken up by hepatocytes if injected by the NTV procedure for several minutes after HTV injection (Zhang *et al.*, 2004). These results indicate that hepatocytes after HTV injection remain in a "competent" state for several minutes. This "competent" state may be due to the persistence of membrane pores or increased vesicular uptake.

Delayed cellular entry has also been observed using fluorescently labeled pDNA (Budker *et al.*, 2000). At less than 5 min after HTV injection, fluorescently labeled pDNA was located at the plasma membrane of hepatocytes and was associated with sinusoidal cells. At 1 h after HTV, the labeled pDNA was inside of more than 10% of the hepatocytes (Eastman *et al.*, 2002). This delay in hepatocyte uptake could be due to the slow transport of DNA through membrane pores or uptake into vesicles. With other fluorescently labeled molecules, such as proteins and siRNA, delayed uptake was less apparent (V. Budker *et al.*, manuscript in preparation). Another study using a different DNA fluorescent-labeling method did not observe increased hepatocyte uptake after HTV injection (Kobayahi *et al.*, 2001).

V. ENTRY OF NUCLEIC ACID INTO THE NUCLEUS

All eukaryotic cells are divided into two main functionally distinct, membrane-bound compartments: the cytoplasm and nucleus. The two compartments are separated by the nuclear envelope—two concentric membrane layers punctured by pores. The pores, called nuclear pore complexes (NPCs), are formed by gigantic supramolecular assemblies of multiple copies of some 30–50 different proteins (Fahrenkrog and Aebi, 2003). NPCs allow the selective, active transport of macromolecules in both directions, provided they carry specific signals, or "addresses," that are recognized by receptor molecules, which in turn mediate their translocation through the central channel of the pore. Macromolecules larger than 50–60 kDa cannot cross this barrier efficiently without displaying such signals. This is why oligonucleotides can enter the nucleus readily after microinjection into the cytoplasm of cultured cells or after HTV injection. Once in the nucleus they are retained by interaction with positively charged chromatin components and thereby accumulate in the nucleus.

In contrast, the nuclear entry for much larger sized pDNA is more challenging. Cytoplasmic microinjections injections in asynchronized, cultured cells required 50- to 250-fold higher concentrations of pDNA than nuclear injections did to achieve similar levels of expression (Ludtke *et al.*, 2002). In addition, approximately 0.6 and 2.9 molecules injected into the nucleus enabled 10 and 50% of the cells to express detectable levels of GFP, respectively. This

indicates that only a few pDNA molecules are necessary to achieve expression in a cell, thus greatly increasing the difficulty of studying the cellular transport steps necessary for foreign gene expression.

Our study also found that pDNA was expressed at reasonably high levels following its microinjection into the cytoplasm of HeLa, BHK 21, and 3T3 cells that had not divided (Ludtke *et al.*, 2002). For example, after 10 and 1000 ng/μl of pDNA were injected cytoplasmically, 28 and 50% of the cells that had not divided expressed GFP, respectively, as compared to 50 and 90% for cells that had divided. This result suggested that pDNA could enter the nonmitotic nuclei of mononucleated cells, albeit at a lower efficiency than mitotic nuclei. Other studies using different protocols have observed that cell division enhanced cytoplasmic-injected pDNA expression up to 15-fold (Escriou *et al.*, 2001; Jiang *et al.*, 1998). The ability of pDNA to enter intact nuclei of nondividing cells is consistent with our previous experience using multinucleated myotubes and digitonin-permeabilized cells in culture (Hagstrom *et al.*, 1997; Wolff *et al.*, 1992). Also consistent with these observations is a report on the nuclear accumulation of long, linear λ DNA molecules in reconstituted Xenopus nuclei (Salman *et al.*, 2001). The final conclusion of that paper is that the DNA enters the nucleus through the NPC, without using any energy source, but with very slow kinetics. The first step is especially slow until the DNA end becomes associated with a NPC and starts to thread through it. Then there is a slow inward movement that is thought to be driven by linear diffusion and a weak "pulling force" from inside, which may be generated by retention of the end inside (Salman *et al.*, 2001). The measured femto-newton force range is two orders of magnitude smaller than the pico-newton forces associated with typical motor protein activities, thus it seems very unlikely that a molecular motor mechanism was involved in the translocation step.

These studies in cultured cells are relevant to several observations concerning pDNA delivery to hepatocytes *in vivo*. For one, is hepatocyte mitosis required for pDNA expression *in vivo*? After portal vein injections, nuclei with BrDU incorporation increased to approximately 1% of the hepatocytes 2 days after injection but remained near basal levels of 0.1% 6 h and 1 day after injection (Herweijer *et al.*, 2001). Given that the percentage of transfected hepatocytes is substantially greater than the percentage of dividing cells, it is unlikely that hepatocyte mitosis is required for pDNA to enter the nucleus from the cytoplasm.

The transport of pDNA through the cytoplasm may also be limiting. The disparity between the ability for naked DNA to enter isolated nuclei and the inefficiency of its nuclear entry from the cytoplasm of intact cells can be explained by structural and biochemical differences between the reconstituted nuclei in a cell-free environment and the complex membrane and cytoskeletal network present in the intact cytoplasm. For example, addition of a cytoplasm

extract decreases the entry of naked DNA into the nuclei of digitonin-treated cells (Hagstrom *et al.*, 1997). This phenomenon of "cytoplasmic sequestration" was observed even after heavily labeling the pDNA with hundreds of NLS peptides (Sebestyen *et al.*, 1998). The nature of the molecular interactions leading to this cytoplasmic retention has not been fully characterized yet. Furthermore, pDNA that is held up in the cytoplasm can be degraded by cytoplasmic DNases (Lechardeur and Lukacs, 2002). After HTV injection, many of the hepatocytes are swollen. Perhaps this attenuates the injected sequestration or degradation of the pDNA in the cytoplasm (Zhang *et al.*, 2004). Consistent with this hypothesis is the observation that expression from cytoplasmically microinjected pDNA increases when it is injected within a larger volume (Zhang *et al.*, 2004).

An alternative possibility is that the pDNA enters the nucleus by a more direct route. We have observed labeled pDNA in the nucleus 5 min after HTV injection (V. Budker *et al.*, manuscript in preparation) (Fig. 1.2B). The time course for gene expression may have some relevance to this question of pDNA nuclear transport after HTV injection. The onset of expression was more rapid after the nuclear microinjection of pDNA than after the cytoplasmic injection (Ludtke *et al.*, 2002). The observation that expression from a CMV-LacZ construct appeared within 2 h after HTV injection and peaked at 8 h (Rossmanith *et al.*, 2002) also raises the possibility of a more direct route to the nucleus. One possibility is that HTV causes a convective flow into the cytoplasm and breaches the nuclear barrier transiently.

VI. UPTAKE OF NAKED DNA BY MUSCLE CELLS AFTER DIRECT INTRAMUSCULAR INJECTION

The following section highlights several features of naked pDNA expression in muscle that are of relevance to the mechanism of entry. After the direct injection or intravascular delivery of naked pDNA, foreign gene expression is mainly limited to myofibers (Hagstrom *et al.*, 2004; Wolff *et al.*, 1990). It is noteworthy that pDNA expression only occurs in the parenchymal cells of either muscle or liver, despite the fact that nonparenchymal cells (such as endothelial cells) come into contact with the pDNA and the high pressure and increased flow. In addition, all types of striated muscle, including both type I and type II skeletal myofibers and cardiac muscle cells, can express naked pDNA, indicating that the uptake process is common among all types of striated muscles (Acsadi *et al.*, 1991; Jiao *et al.*, 1992).

Muscles such as the rectus femoris or tibialis anterior that are circumscribed by a well-defined epimysium may enable the highest levels, as they provide the best distribution and retention of the injected pDNA. Similarly,

high levels of expression have been observed in diaphragm muscle, a well-marcated thin muscle (Davis and Jasmin, 1993; Liang *et al.*, 2004; Liu *et al.*, 2001). Expression can also be aided by enhanced distribution of the pDNA, which has been accomplished by preinjection of muscles with large volumes of hypertonic solutions and polymers or by an improved injection technique, such as positioning the needle along the longitudinal axis of the muscle (Davis *et al.*, 1993; Mumper *et al.*, 1996). Taken together, these results suggest that expression is enhanced if the muscle can be swelled during direct, intramuscular injection. However, implantation with forceps of pellets of dried pDNA also yields high expression (Jiao *et al.*, 1992; Wolff *et al.*, 1991). Also, myofibers distant from the implantation site are often transfected, suggesting that muscle swelling is not absolutely necessary for expression.

The state of muscle appears to affect the levels of expression. High levels of foreign gene expression were obtained in 2-week-old mouse and rat muscles (Danko *et al.*, 1997). For example, approximately 50% of the myofibers were intensely blue following the intramuscular injection of a β-galactosidase expression vector in 2-week-old Balb/C mice. Also, muscle regeneration induced by myotoxic agents enabled higher levels of expression (Danko *et al.*, 1994; Vitadello *et al.*, 1994; Wells and Wells, 2002). Muscle regeneration induced by ischemia also enhanced pDNA expression (Takeshita *et al.*, 1996; Tsurumi *et al.*, 1996). Myotoxic agents such as cardiotoxin and amide local anesthetics such as bupivacaine (Marcaine) are advantageous because they selectively destroy myofibers without harming myoblasts or the vascular endothelial cells, thus enabling complete recovery of the muscle. Optimal luciferase expression was obtained when the pDNA was injected 3 to 7 days after bupivacaine or cardiotoxin injection or initiation of ischemia, a time when a substantial number of muscle cells have begun to recover from the effects of the myotoxic agent. In these studies involving young muscle or regenerating muscle, increased foreign gene activity may be due to either enhanced pDNA uptake or expression such as from transcriptional activation.

A general trend is that expression decreases as the size of the animal increases. Expression is slightly less in rats than in mice but is substantially less in animals larger than rodents such as rabbits, cats, and monkeys (Jiao *et al.*, 1992). This was observed in both younger and adult larger animals, including nonhuman primates. The connective tissue in primate muscle may prevent the distribution of the pDNA or its contact with sarcolemma. Histochemical and fluorescent stains indicated that primate muscle had substantially more connective tissue within the perimysium. The thicker perimysium in primate muscle may restrict the distribution of the pDNA or may serve as a conduit and an increased potential space for the pDNA to be dispersed without coming in contact with the myofibers.

While expression following liver delivery is rapid, the onset of expression in muscle appears slower. In muscle, expression from RNA vectors peaks within the first day after injection, whereas expression from pDNA vectors peaks at 14 days or even longer (Davis *et al.*, 1993; Manthorpe *et al.*, 1993; Wolff *et al.*, 1990, 1992). This could be the result of delayed pDNA uptake or transcriptional expression. Southern blot analysis indicates that the majority of the injected pDNA is degraded rapidly within hours but that a small percentage of the injected pDNA persists in an open, circular form (Manthorpe *et al.*, 1993; Wolff *et al.*, 1991). Increased expression is obtained with increasing amounts of pDNA, but expression plateaus at different amounts of injected pDNA depending on the muscle type, species, and pDNA vector.

The state of the injected DNA also affects expression. Injection of linearized pDNA yields much less expression, presumably due to its enhanced degradation (Buttrick *et al.*, 1992; Wolff *et al.*, 1992). Larger sized pDNA express less efficiently on a molar basis when injected intramuscularly (or intravascularly) into muscle but less so in liver when injected using HTV injections (Zhang *et al.*, 2004). These results suggest that differences exist in the upake of naked DNA by myofibers as compared to hepatoctyes.

VII. DELIVERY OF NAKED DNA TO MUSCLE VIA INTRAVASCULAR ROUTES

Given that the expression of naked DNA was inefficient in larger animals and humans, efforts were sought to increase expression. The intravascular delivery of naked pDNA to muscle cells was attractive because it avoids the limited distribution of pDNA though the interstitial space following intramuscular injection. Muscle has a high density of capillaries (Browning *et al.*, 1996) that are in close contact with the myofibers (Lee and Schmid-Schonbein, 1995). Delivery of pDNA to muscle via capillaries puts the pDNA into direct contact with every myofiber and substantially decreases the interstitial space the pDNA has to traverse in order to access a myofiber. However, the endothelium in muscle capillaries is of the continuous, nonfenestrated type and has low solute permeability, especially to large macromolecules. This is in contrast to the fenestrated endothelium of liver.

Thus, it was surprising that high levels of expression can be achieved following the intravascular injection of naked pDNA to limb and diaphragm muscles via either artery or venous routes of administrations (Hagstrom *et al.*, 2004; Liang *et al.*, 2004). There is critical dependence on the volume and speed of injection, suggesting that increased hydrostatic pressure, rapid flow, or both are required for efficient expression. Use of fluorescently labeled DNA provided direct evidence that these injection conditions enabled extravasation of the

injected DNA (Budker *et al.*, 1998). Muscle swelling appears to correlate with expression levels.

In order to understand the mechanism by which pDNA can be extravasated, it is instructive to consider the mechanisms by which smaller macromolecules such as proteins can traverse the endothelial barrier. One postulated mechanism is transcytosis, which can utilize plasmalemmal vesicles or transient transendothelial channels formed by the fusion of vesicles. These anatomical features may be responsible for the physiologic transcytosis observations modeled by a large number of small pores with radii of about 4 nm. Physiology experiments also suggest that muscle endothelium has a very low number of large pores with radii of 20–30 nm. Although the radius of gyration of 6 kb pDNA is ~100 nm (Fishman and Patterson, 1996), supercoiled DNA in plectonomic form has superhelix dimensions of approximately 10 nm (Rybenkov *et al.*, 1997). This implies that pDNA is capable of crossing microvascular walls by stringing through the large pores. Presumably, the rate of pDNA extravasation is increased by enhancing fluid convection through these large pores by raising the transmural pressure difference in selective regions.

VIII. CONCLUSIONS

As is apparent from this review, studies to elucidate the mechanism of naked pDNA entry into cells have been quite stimulating. It remains remarkable that naked DNA can in fact be expressed efficiently in a variety of cells *in vivo*. Given that viruses have evolved over millions of years of evolution to accomplish DNA transfer, it is surprising that just naked DNA can in fact traverse the several steps in the pathway to expression. Most likely naked DNA delivery did not evolve specifically for polynucleic acid uptake. Instead the pDNA may exploit a transport system that evolved for other purposes. For example, polynucleic acid uptake may provide an evolutionary advantage on the basis of its exquisite ability for immune activation. The conditions required for optimal naked DNA uptake, such as tissue swelling, may mimic the tissue conditions associated with infection. DNA uptake may also play a role in clearing the large amount of released polynucleic acid that occurs physiologically and in disease states.

The ability for naked DNA to enter cells may also have implications for the evolution of viruses. It casts the ability of viruses in a different light; their accomplishment—the ability to transduce cells—may not have been so remarkable.

As further studies continue to elucidate the mechanisms of nucleic acid transport, one can look forward to greater insight into this very interesting phenomenon. These efforts are also likely to lead to improved methods that enable greater expression efficiencies and have greater clinical utility. The

eventual impact will be on more powerful gene therapies to treat disease and to alleviate suffering.

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Gene Therapy (1998) 5, 272–276

BRIEF COMMUNICATION

The efficient expression of intravascularly delivered DNA in rat muscle

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Previous studies have demonstrated that muscle can take up and express naked DNA or RNA. This study demonstrates that the pDNA can be delivered to and expressed within skeletal muscle when injected rapidly, in a large volume and when all blood vessels leading into and out of the hindlimb are occluded. The additional use of collagenase, papaverine and ischemia raised expression moderately but was not critical. These results demonstrate that a nonviral method can lead to high levels of expression in the muscles of adult animals larger than mice.

Keywords: *gene transfer; gene therapy; muscle; plasmid DNA; endothelium*

The direct transfer of genes into humans is an attractive approach for gene therapy because it avoids laborious and costly cell culture. Nonviral or viral vectors have been administered either intravascularly or intraparenchymally. In adult animals, the intravascular delivery of adenoviruses or cationic lipid–DNA complexes mostly results in expression in vascular-accessible cells such as endothelial cells or hepatocytes reached via the sinusoid fenestrae.[1,2]

We have previously shown that muscle can take up and express naked DNA or RNA.[3] More recently, we have found that naked plasmid DNA (pDNA) is expressed in hepatocytes when injected into the portal vein under increased osmotic and hydrostatic pressure which widens the sinusoid fenestrae and enhances pDNA extravasation.[4] The levels of pDNA expression in the liver were orders of magnitude greater than we had previously achieved from intramuscular injection of naked pDNA.

The intravascular delivery of naked pDNA to muscle cells is desirable. Muscle has a high density of capillaries[5] that are in close contact with the myofibers.[6] However, the endothelium in muscle capillaries is of the continuous, non-fenestrated type and has low solute permeability, especially to large macromolecules.[7] The mechanism of macromolecule transendothelial transport is poorly understood. Cell biologists have proposed that it occurs by transcytosis involving plasmalemmal vesicles or by convective transport through transient transendothelial channels formed by the fusion of vesicles.[8] Physiology experiments suggest that the muscle endothelium has a large number of small pores with radii of about 4 nm and a very low number of large pores with radii of 20–30 nm.[9] Although the radius of gyration of 6 kb pDNA is approximately 100 nm,[10] supercoiled DNA in plectonomic form has superhelix dimensions of approximately 10 nm.[11] This implies that pDNA is capable of crossing microvascular walls by stringing through the large pores. We hypothesized that the rate of pDNA extravasation could be increased by enhancing fluid convection through these large pores by raising the transmural pressure difference in selective regions. This report demonstrates that intravascular pDNA injections under high pressure can in fact lead to high levels of foreign gene expression in muscles throughout a selected hindlimb of an adult rat.

Four hundred and seventy-five micrograms of pCILux in normal saline solution (NSS) were injected into the femoral arteries of adult Sprague–Dawley rats while blood inflow and outflow were blocked (Figure 1). Injection of pCILux, a luciferase expression vector utilizing the CMV promoter (constructed by inserting the luciferase gene (*Hin*dIII–*Bam*HI fragment from pBlueCMVLux) into the *Sma*I site of pCI) was done after both the femoral artery and vein were occluded for 10 min. Two days after the pDNA injections, the luciferase activity was measured in all the muscles of the hindlimb (Figure 2). The highest level of luciferase expression was achieved when the pCILux was injected in 9.5 ml of normal saline within 10 s. Injection volumes less than 9.5 ml resulted in substantially lower expression (Figure 2a). Injection times more than 10 s also resulted in much less expression (Figure 2b). This critical dependence on the volume and speed of injection suggests that either increased hydrostatic pressure and rapid flow or both are required for efficient expression. Artery injections performed without occluding the femoral vein resulted in approximately 200-fold less expression (1.8 ± 1.2 ng of luciferase per all hindlimb muscles).

Further studies were performed to determine the effect of ischemia on the expression of the intravascularly injected pCILux. The degree of ischemia was adjusted by varying the time during which both the femoral artery and vein were occluded before pDNA injection (Figure 2c). Although the highest level of expression was obtained with 10 min of ischemia, the expression levels

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Received 24 June 1997; accepted 24 September 1997

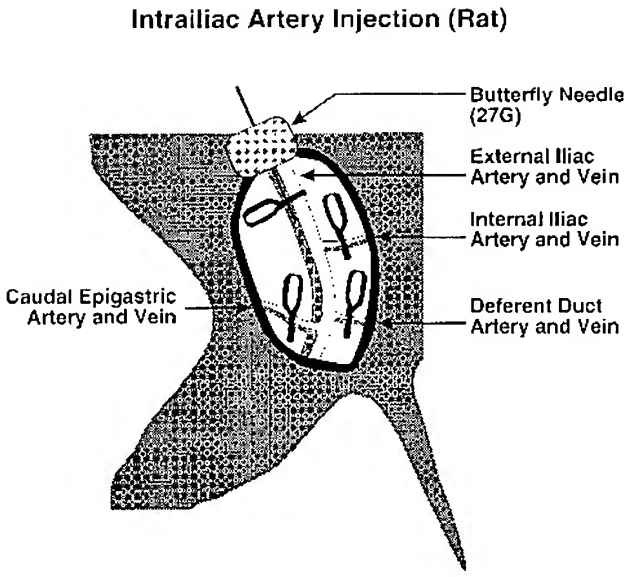

Figure 1 A 4 cm long abdominal midline excision was performed in 150–200 g adult Sprague–Dawley rats anesthetized with 80 mg/mg ketamine and 40 mg/kg xylazine. Microvessel clips (Edward Weck, Research Triangle Park, NC, USA) were placed on external iliac, caudal epigastric, internal iliac and deferent duct arteries and veins to block both outflow and inflow of the blood to the leg. A 27 G butterfly needle was inserted into the external iliac artery and the DNA solution was injected by hand.

were only a few fold lower at shorter or longer ischemia times. This suggests that ischemia is not a critical factor for enabling egress of the pDNA out of the intravascular space. However, the blood flow for the zero ischemia time-point is disrupted for approximately 30 s and this may be sufficient to affect vascular permeability. Ischemia could increase expression either by capillary recruitment and vasodilatation or by augmenting permeability.[12] In addition, ischemia could increase pDNA expression by affecting transcription or translation. Ischemia can be tolerated by muscle for 2 to 3 h.[13] Histologic analysis of the muscle did not reveal any hemorrhage or myofiber damage.

Other factors were explored to increase the level of expression; hypotonic (Figure 3, condition 2) or hypertonic (Figure 3, condition 3) injection solutions resulted in lower expression. The effect of the hypertonic injection solution (NSS with 15% mannitol) may have been in part obscured by the slower rate of injection (over 30 s instead of 10 s) caused by the increased viscosity of the solution. The pre-injection with collagenase resulted in a 3.5-fold increase to 1332 ng per total muscles (Figure 3, condition 4). The collagenase was used to disrupt the basement

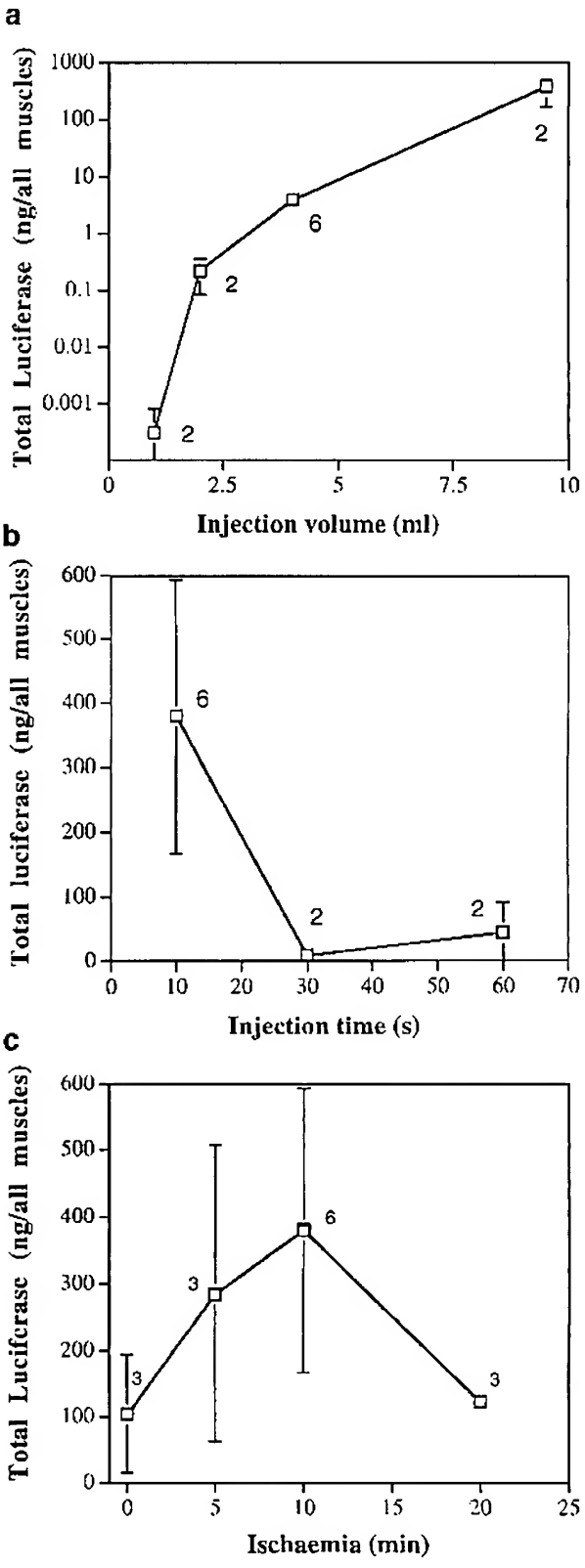

Figure 2 Effects of injection solution volume (a), injection time (b) and preinjection ischemia time (c) on the mean levels of luciferase expression in rat hindlimb muscles. Two days after 475 μg of pCILux in 9.5 ml of NSS were injected unilaterally into the iliac artery of adult Sprague–Dawley rats, luciferase activities in six hindlimb muscle groups (anterior upper leg, medial upper leg, posterior upper leg, anterior lower leg, posterior lower leg and foot) were measured as previously reported and presented as the means of the total luciferase levels of all six muscle groups. The pre-injection ischemia time was 10 min in (a) and (b). Numbers adjacent to data points indicate the number of animals (one limb per animal) assayed for each condition. Error bars indicate the standard error.

Table 1 Distribution of luciferase expression among the different hindlimb muscle groups 2 days following intra-arterial injection with 475 μg of pCILux in 9.5 ml of NSS over 10

Injection type	*Muscle group*	*Total luciferase (ng)*	*Luciferase concentration (ng/g tissue)*
Intravascular	Upper leg anterior	149.1 ± 51.2	109.2 ± 37.5
	Upper leg posterior	74.6 ± 36.0	43.4 ± 20.9
	Upper leg medial	88.7 ± 63.7	61.6 ± 44.5
	Lower leg posterior	114.5 ± 89.7	66.0 ± 51.7
	Lower leg anterior	3.0 ± 1.0	5.4 ± 1.7
	Foot	0.5 ± 0.2	4.1 ± 1.4
Intramuscular	Upper leg anterior	3.7 ± 0.9	2.8 ± 0.8

Means and their standard deviations are shown for six animals.

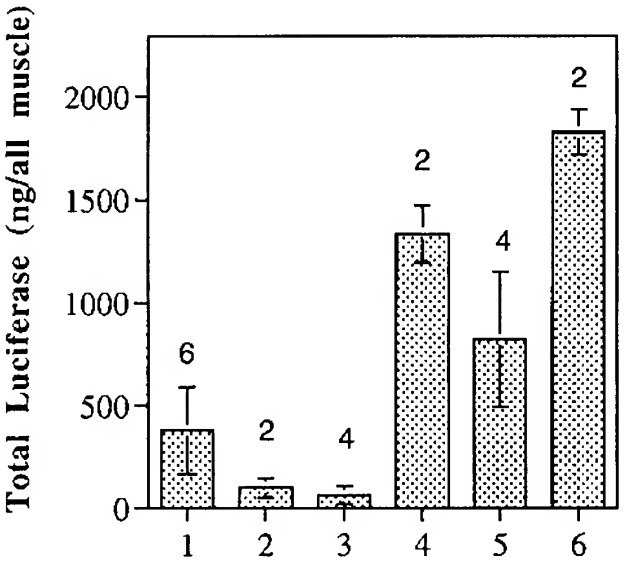

Figure 3 Effects of various conditions on luciferase expression in rat hindlimb muscles following the injection of pCILux DNA into the iliac artery. Condition 1, 475 μg DNA in 9.5 ml of NSS were injected within 10 s after a 10-min occlusion of limb blood flow; Condition 2, 475 μg DNA in 9.5 ml of H_2O were injected within 10 s after a 10-min occlusion of limb blood flow; Condition 3, 475 μg DNA in 9.5 ml of NSS with 15% mannitol were within 30 s after a 10-min occlusion of limb blood flow; Condition 4, 80 μg of collagenase in 1 ml of NSS were injected into iliac artery immediately after blood flow occlusion. After 10 min, blood flow was opened for several seconds, closed again, and 475 μg DNA in 9.5 ml of NSS were injected within 10 s. Condition 5, 500 μg of papaverine (Sigma, St Louis, MO, USA) in 3 ml of NSS were injected into the iliac artery immediately after blood flow occlusion. After 5 min, blood flow was opened for several seconds, closed again, and 475 μg DNA in 9.5 ml of NSS were injected within 10 s. Condition 6, 40 μg of collagenase and 500 μg of papaverine in 3 ml of NSS were injected into the iliac artery immediately after blood flow occlusion. After 5 min, blood flow was opened for several seconds, closed again, and 475 μg DNA in 9.5 ml of NSS were injected within 10 s. Bold numbers indicate the number of animals assayed at each set of experimental conditions. Error bars indicate the standard error.

membrane of the capillaries and thereby increase pDNA egress. It could have also increased expression by disrupting the extracellular matrix within the muscle.

Pre-injection with papaverine, a vasodilator, also caused a small increase in expression (Figure 3, condition 5). The highest levels were obtained when the legs were pre-injected with both papaverine and collagenase (Figure 3, condition 6). Forty micrograms of collagenase per ml was used in condition 6 because the combination of 80 μg collagenase per ml with papaverine resulted in some hemorrhage. Further studies are in progress to determine the optimal conditions for collagenase and papaverine administration without causing hemorrhage.

Luciferase expression was observed in all muscle groups in the leg (Table 1). The reduced levels of expression in the lower anterior leg and foot are probably due to the high content of tendons and small muscles in this region. The total amount of luciferase expressed following intravascular injection was up to 40 times higher than the levels following intramuscular injection.

The type and percentage of the transfected cells in the muscle were determined using the β-galactosidase reporter system (Figure 4). The vast preponderance of the β-galactosidase-positive cells were myofibers. Very few endothelial cells were stained blue. With the best injection condition (condition 6 in Figure 3), up to 50% of myofibers expressed β-galactosidase in many areas of the muscles. More quantitative analysis was performed by counting the percentage of positive cells in proximal, middle and distal cross-sections for each of the five muscle groups in four animals (Table 2). Approximately 1000 myofibers per section were evaluated in an unbiased manner by counting the number of unstained and stained myofibers within a grid box (containing a total of approximately 30 cells) that was moved continuously along the central vertical and horizontal parts of each section. In the upper anterior leg muscle group, 11 to 21% of the myofibers were positively stained. Similarly high percentages were observed in the lower posterior leg muscle group. Approximately half the percentage of cells were positive in the posterior and medial upper leg muscle groups. The percentages of positive cells were substantially less in the lower anterior muscle group. Of the 72 126 myofibers counted in all the sections in all the muscle groups of all four animals, 10.1% were β-galactosidase-positive.

These expression results provide indirect evidence that pDNA extravasation occurred. More direct evidence was obtained using fluorescently labeled DNA injected into the femoral artery (condition 1 in Figure 3). The labeled DNA was distributed extravascularly in all the limb muscles and surrounded most of the myofibers (data not shown).

In conclusion, these results demonstrate that the intra-arterial delivery of pDNA to muscle can be greatly

Table 2 Percentage of myofibers expressing β-galactosidase among the different hindlimb muscle groups 2 days following intra-arterial injection with 475 μg of pCILacZ per condition 6 in Figure 3

Muscle group	*Percentage of myofibers expressing β-galactosidase*				
	Animal 1	*Animal 2*	*Animal 3*	*Animal 4*	*Mean*
Upper leg anterior	11.1	14.6	14.7	21.2	15.4
Upper leg posterior	3.3	1.4	15.2	9.7	7.4
Upper leg medial	9.8	5.7	9.8	9.8	8.8
Lower leg posterior	15.3	2.4	12.2	19.5	12.4
Lower leg anterior	0.9	1.1	3.0	1.5	1.6

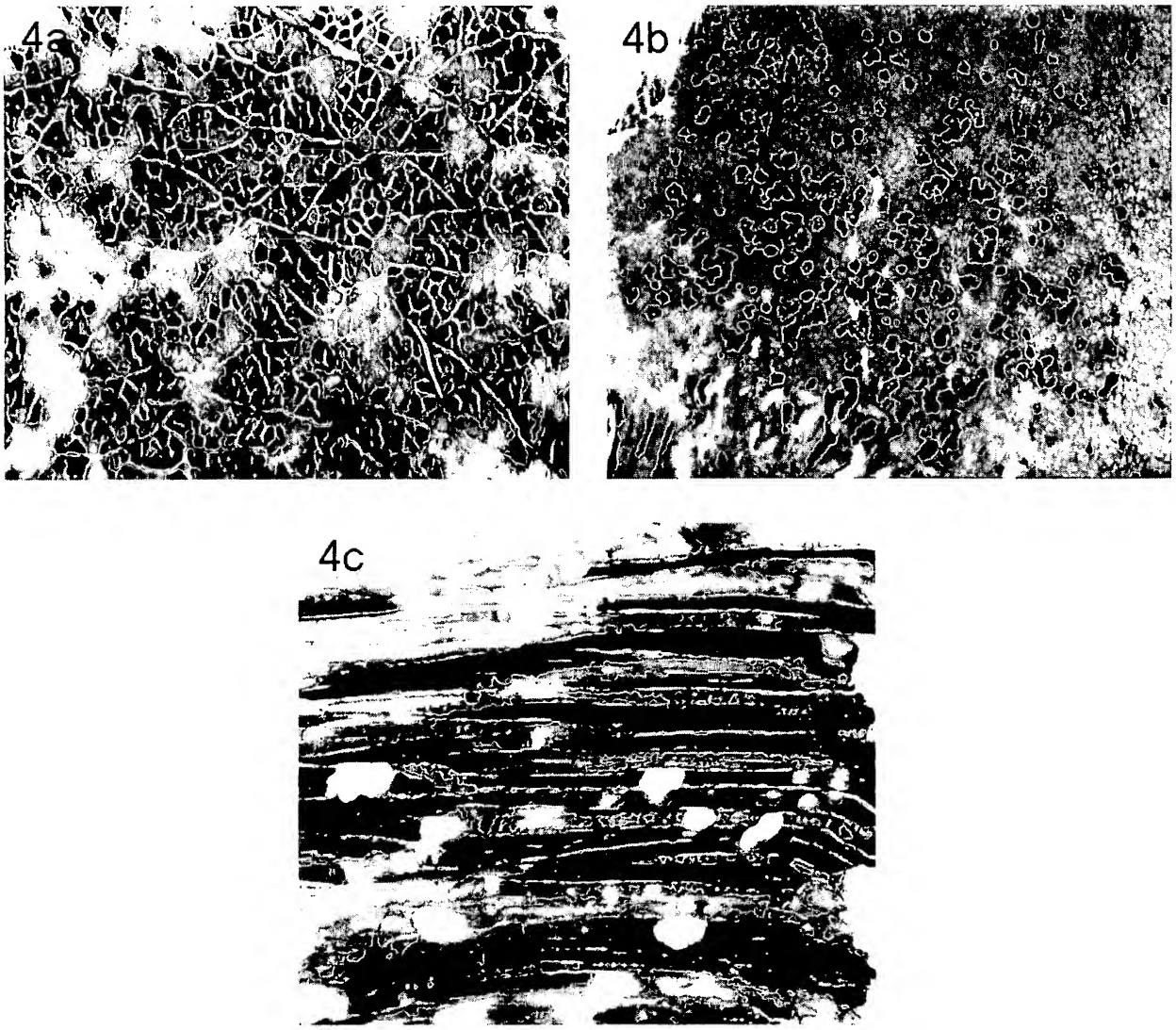

Figure 4 Histochemical analysis of β-galactosidase expression in rat hindlimb muscles 2 days after the intra-arterial injection of 475 μg of pCILacZ (pCILacZ was constructed by placing the E. coli LacZ gene (PstI–ApaI fragment pBS-RSV-LacZ) into the pCI vector (SmaI site)) using condition 4 in Figure 3. Muscle sections, 15-μm thick, were stained overnight using X-gal at room temperature and counterstained with eosin.[16] (a) Muscle cross-section from posterior lower leg with a high expression level; (b) Muscle cross-section from anterior upper leg with a low expression level; (c) Longitudinal section from posterior lower leg. Magnifications were × 160 for a and c and × 100 for b.

enhanced when injected rapidly, in a large volume, and with all blood vessels leading into and out of the hindlimb occluded. These conditions presumably increase the intravascular hydrostatic pressure and thereby increase the convective outflow which brings the pDNA into contact with myofibers. The high intravascular pressure may increase the number, size and permeability of the microvascular pores.[14,15] Preliminary studies using collagenase suggest that enzymatic disruption of the vessel's basement membrane or muscle extracellular matrix may increase the delivery of pDNA to the myofibers. Ischemia and papaverine also increases expression moderately.

This study shows that intravascular injection increases expression of DNA in muscle up to 40-fold as compared with intramuscular injection. This is the first demonstration of the efficient expression of naked plasmid DNA in the muscles of adult animals larger than mice. It is also the first report of an intravascular nonviral gene transfer approach for muscle. Further studies in larger animals will determine the clinical relevance of this study.

Acknowledgements

This research was supported in part by funding from the Muscular Dystrophy Association (USA). Special acknowledgement to Daniel Bucker who serves as an inspiration for our work.

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Gene Therapy (2006), 1–10

www.nature.com/gt

ORIGINAL ARTICLE

Plasmid-mediated VEGF gene transfer induces cardiomyogenesis and reduces myocardial infarct size in sheep

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*We have recently reported that in pigs with chronic myocardial ischemia heart transfection with a plasmid encoding the 165 isoform of human vascular endothelial growth factor (p*VEGF$_{165}$*) induces an increase in the mitotic index of adult cardiomyocytes and cardiomyocyte hyperplasia. On these bases we hypothesized that* VEGF *gene transfer could also modify the evolution of experimental myocardial infarct. In adult sheep p*VEGF$_{165}$ *(3.8 mg,* n = *7) or empty plasmid (*n = *7) was injected intramyocardially 1 h after coronary artery ligation. After 15 days infarct area was 11.3*± *1.3% of the left ventricle in the VEGF group and 18.2*± *2.1% in the empty plasmid group (*P < *0.02). The mechanisms involved in infarct size reduction (assessed in additional sheep at 7 and 10 days after infarction) included an increase in early angiogenesis and arteriogenesis, a decrease in peri-infarct fibrosis, a decrease in myofibroblast proliferation, enhanced cardiomyoblast proliferation and mitosis of adult cardiomyocytes with occasional cytokinesis. Resting myocardial perfusion (*^{99m}*Tc-sestamibi SPECT) was higher in VEGF-treated group than in empty plasmid group 15 days after myocardial infarction. We conclude that plasmid-mediated* VEGF *gene transfer reduces myocardial infarct size by a combination of effects including neovascular proliferation, modification of fibrosis and cardiomyocyte regeneration.*
Gene Therapy advance online publication, 6 April 2006; doi:10.1038/sj.gt.3302708

Keywords: *myocardial infarction; VEGF; heart regeneration; cardiomyocytes*

Introduction

Despite that the prognosis of patients with coronary artery disease has improved with the incorporation of new drugs and reperfusion strategies, heart failure resulting from myocardial infarction with extensive cardiomyocyte loss has an ominous outcome. For this reason, therapeutic interventions tending to reduce infarct size are needed.

Recently, in an animal model of chronic myocardial ischemia we reported that administration of plasmid encoding recombinant human vascular endothelial growth factor $(VEGF)_{165}$ ($pVEGF_{165}$) induces a significant increase in the mitotic index[1] and hyperplasia[2] of adult cardiomyocytes. Therefore, we hypothesized that VEGF could display a beneficial effect on acute myocardial infarction (AMI) by promoting cardiomyogenesis. We thus studied in adult sheep the effect of human $VEGF_{165}$ gene transfer on the evolution and size of AMI during the first 2 weeks after acute, permanent coronary artery occlusion.

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Received 20 July 2005; revised 17 November 2005; accepted 18 November 2005

Results

Vascular endothelial growth factor reduces infarct size

Determination of infarct size was carried out on 26 adult sheep with AMI achieved by ligating the left anterior descending (LAD) artery at its distal third. At 1 h after LAD occlusion, the animals were randomized to receive 3.8 mg of a plasmid encoding the human $VEGF_{165}$ gene ($pVEGF_{165}$, $n = 13$) or empty plasmid (placebo, $n = 13$), in 10 intramyocardial injections distributed along the peri-infarct area. The nature of the injectates was kept blind until the end of data processing. Twelve animals ($n = 6$ per group) were killed at 10 days and 14 ($n = 7$ per group) at 15 days.

In sheep killed at 10 days, AMI size was similar for placebo (17 ± 1.8% of the left ventricle) and VEGF groups (18.7 ± 3.7%, P = NS), but in sheep killed at 15 days it was significantly smaller for VEGF group (placebo: 18.2 ± 2.1%, VEGF: 11.3 ± 1.3%, $P < 0.02$, Figure 1). A similar result was observed for absolute values of infarct area (10 days: placebo 9.7 ± 0.9 cm², VEGF 11.6 ± 1.6, P = NS; 15 days: placebo 9 ± 1, VEGF 6.1 ± 0.8, $P < 0.05$).

Vascular endothelial growth factor enhances adult cardiomyocyte division

On account that cardiomyocyte mitosis is known to occur after AMI[3] and that *VEGF* gene transfer induces entrance

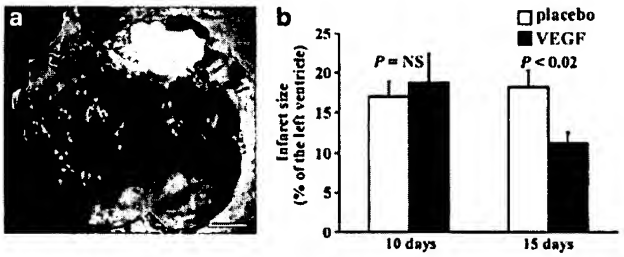

Figure 1 Infarct aspect and size. (a) At 10 and 15 days after coronary artery occlusion the infarct appears as a white area sharply demarcated from the surrounding myocardium. Bar: 20 mm. (b) Infarct size, as a percentage of left ventricular (LV) area, was smaller in *vascular endothelial growth factor (VEGF)*-treated sheep at 15 but not at 10 days after coronary artery occlusion.

in mitosis and hyperplasia of adult cardiomyocytes in chronic myocardial ischemia,[1,2] we studied the mitotic index (number of mitosis per 10^6 cardiomyocyte nuclei (CMN)) in the 10 rows of viable myocardium bordering the infarct. Although in some reports it is held that confocal microscopy with fluorescent dyes is the gold standard for identifying cardiomyocyte mitosis,[4] we employed Nomarski optics or conventional light microscopy on account that confocal microscopy does not allow to clearly visualize the nuclear envelope. This limitation can lead to confuse true mitosis with endomitosis and/or chromatin fragmentation, as has been reported and illustrated (see Figure 3 of Laguens *et al.*[1] and Figure 1 of Cabeza Meckert *et al.*[5]) in studies using both techniques in chronically ischemic pig hearts and in infarcted human hearts.[1,5] Given the thinness of the tissue sections (half the diameter of a small lymphocyte) and the fact that under Nomarski optics the cell boundaries are readily noticeable, the chance of confusing cycling superimposed cells was unlikely. Adult cardiomyocytes were identified by their size, shape, presence of cross-striations and sarcomeric α-actin.

At 10 days after LAD ligation cardiomyocyte mitotic index was significantly higher in *VEGF*-treated animals (190 ± 27 mitosis/10^6 CMN) than in placebo group (62 ± 10, $P<0.004$, Figure 2a). Examples of cardiomyocytes in different phases of mitosis are shown in Figure 2. Furthermore, we observed occasional images of ongoing cytokinesis (Figure 2h). At 15 days, mitotic activity decreased in the *VEGF*-treated group (VEGF: 47 ± 28 mitosis/10^6 CMN, placebo: 60 ± 30, $P=$NS).

To see whether the presence of AMI is necessary for VEGF to induce cardiomyocyte proliferation, we searched for Ki67+ CMN and mitotic figures in 12 additional sheep with no LAD ligation but injected with p$VEGF_{165}$ ($n=6$) or empty plasmid ($n=6$) in the same zone and with the same doses as in the protocol sheep. Also in this case the nature of the injectates was kept blind until the end of data analysis. After 10 days, neither in placebo nor in *VEGF*-treated sheep cardiomyocyte mitosis or Ki67+ nuclei were found, suggesting that myocardial ischemia is a precondition to allow for the VEGF mitogenic effect.

Vascular endothelial growth factor increases peri-infarct density of cardiomyoblasts

At 10 days after LAD occlusion, small cells (6–10 μm in diameter) with nuclei stained for the Ki67 antigen, positive for sarcomeric α-actin and connexin 43 and negative for smooth muscle actin and Ulex lectin, were present within the area between dead and surviving myocardium. On account of these features they were considered to be cardiomyoblasts.

The *MDR-1*-gene-encoded P170 glycoprotein was found to be positive in cardiomyoblasts, but also in the sarcolemma of peri-infarct adult cardiomyocytes, confirming previous observations.[6] Sca-1 and c-kit immunostainings of cardiomyoblasts were negative because the commercial kits we used did not react with ovine antigens. The same antibodies did react with murine- and human-positive controls. The density of cardiomyoblasts was twofold higher in *VEGF*-treated (1175 ± 141 cells/mm^2) than in placebo sheep (529 ± 86, $P<0.003$). The percent cardiomyoblasts displaying mitotic figures was $12\pm0.8\%$ in *VEGF*-treated sheep and 9.5 ± 2.6 in placebo sheep ($P=$NS). Occasional cytokinesis of these cells (Figure 3) was observed. At 15 days post-AMI cardiomyoblasts were no longer present in either group.

Vascular endothelial growth factor gene transfer reduces myofibroblast proliferation and fibrosis

At 10 days after AMI, in the area between the dead and surviving myocardium, myofibroblasts, identified as smooth muscle actin-positive cells, were less numerous in the VEGF group (5038 ± 513 myofibroblasts/mm^2) than in the placebo group (8513 ± 573, $P<0.002$, Figure 4a). This decrease persisted at 15 days post-AMI (placebo 7280 ± 1431, VEGF 3312 ± 269, $P<0.05$, Figure 4b).

Concordantly, collagen content in the area between dead and surviving myocardium, as calculated from paraffin-embedded sections stained with picrosirius red, was lower in *VEGF*-treated sheep at 10 and 15 days post-AMI (10 days: placebo $70.1\pm1.7\%$ of total area, VEGF $43.5\pm4.5\%$, $P<0.006$; 15 days: placebo $74.3\pm9.8\%$, VEGF: $22.3\pm3.4\%$, $P<0.02$, Figure 4c and d).

Vascular endothelial growth factor increases arteriogenesis in peri-infarct surviving myocardium

At 10 and 15 days post-AMI, small-sized arterioles were clearly visible in the tissue sections stained with an antibody against smooth muscle actin (Figure 5a and b). At 10 days post-AMI *VEGF*-treated sheep showed increased number of arterioles located within 10 rows of peri-infarct viable myocardium (placebo 18.8 ± 5 arterioles/mm^2, VEGF 119.8 ± 32.3, $P<0.03$). After 5 days, the arteriogenic effect of VEGF was preserved (placebo 26.5 ± 7.3, VEGF 83.3 ± 2.9, $P<0.001$).

Vascular endothelial growth factor increases peri-infarct angiogenesis

At 10 and 15 days post-AMI, the fibrosis present between dead and surviving myocardium prevented accurate determination of reparative angiogenesis. This led us to study eight additional sheep with AMI randomized to blindly receive 3.8 mg p$VEGF_{165}$ ($n=4$) or empty plasmid ($n=4$), in order to investigate angiogenesis at 7 days after LAD ligation. We chose this time because it is known that it is about 1 week after AMI when the inflammatory infiltrate subsides and a reparative process starts, with angiogenesis preceding myofibroblast proliferation and fibrosis.[7] Moreover, it was at this time when the product of the transfected *VEGF* gene was

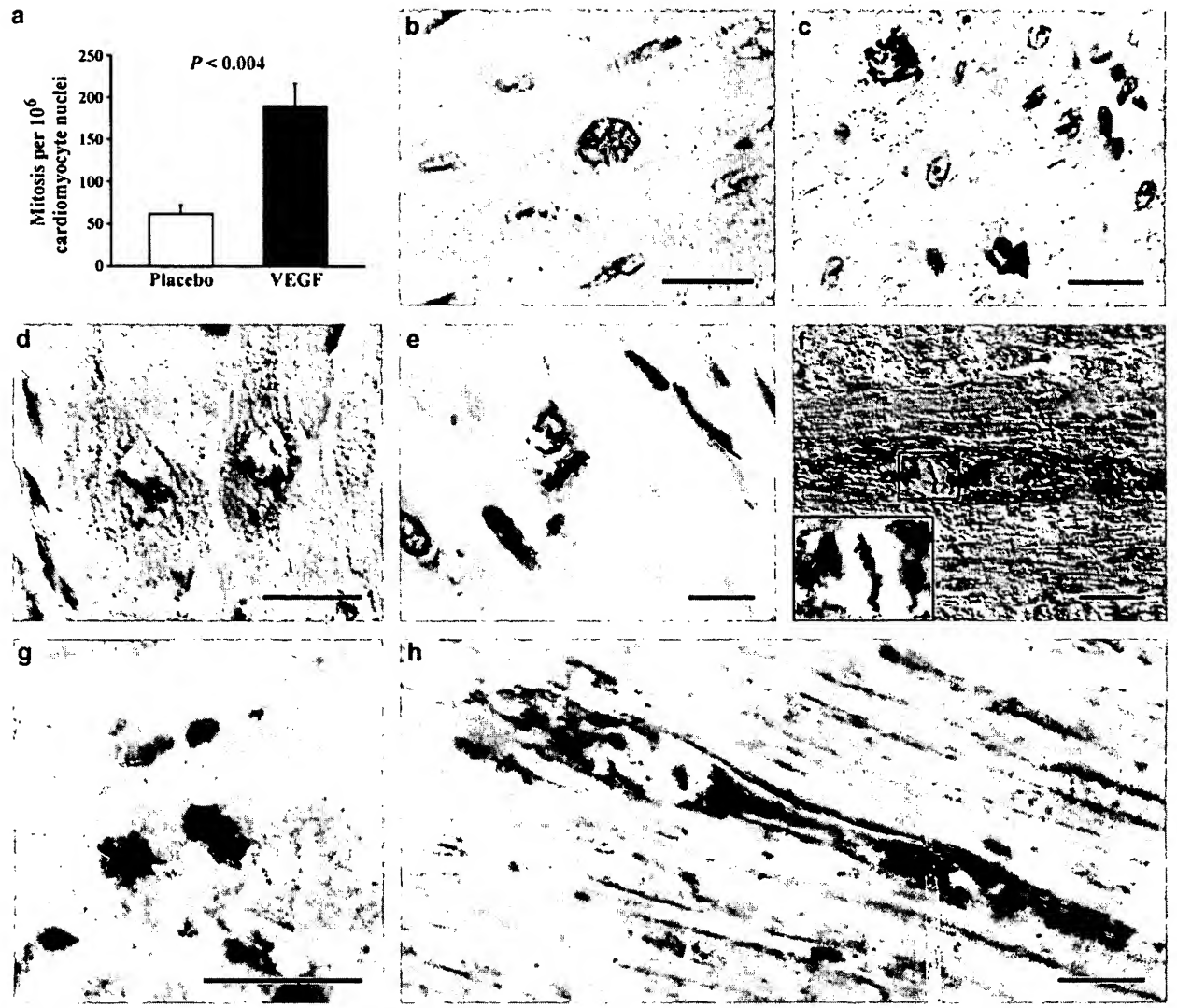

Figure 2 Adult cardiomyocyte mitosis and cytokinesis. (**a**) At 10 days after coronary artery occlusion the mitotic index was higher in *vascular endothelial growth factor (VEGF)*-treated sheep. (**b**) Late prophase. (**c–f**) Metaphase plates in different projections. Occasionally, the metaphase plates were oriented perpendicularly to each other (**e**) and the mitotic spindle, extended between sarcomeric α-actin condensations, was patent (**f**, inset). (**g**) Late anaphase. (**h**) Late telophase with ongoing cytokinesis. (**b–e** and **g**) Anti-Ki67 immunohistochemistry and hematoxylin counterstain. (**f** and **h**) Anti-sarcomeric α-actin immunohistochemistry and hematoxylin counterstain. Bars: 20 μm.

present in the myocardium, as shown by immunohistochemistry and Western blot (see below).

In the area between the remnant of coagulation necrosis tissue and viable myocardium a marked angiogenic response with formation of a network of closely packed small capillaries was observed. Since in our material search of CD34 and von Willebrand factor for identification of endothelial cells with commercial antibodies yielded erratic results, probably because these antibodies are developed to react with other mammalian species, we employed biotinilated Ulex lectin and avidin–peroxidase stain for endothelial cell identification. Ulex lectin specifically binds α-L-fucosyl residues on the endothelial cell membrane and is usually employed for specific identification of these cells.[8] Quantification after Ulex lectin staining (Figure 5c and d) showed that capillary density was significantly higher in VEGF (1805 ± 221 capillaries/mm^2) than in placebo group (703 ± 35, $P < 0.04$).

Vascular endothelial growth factor improves myocardial perfusion

In the animals killed at 15 days post-AMI ($n = 14$), resting myocardial perfusion and ventricular function were studied with gated single-photon emission computed tomography (G-SPECT) 2 days before surgery, 2 days after AMI and just before killing (Figure 6). In one *VEGF*-treated sheep the myocardial perfusion images had unacceptable quality. Therefore, VEGF group results are based on six animals. Basal, preocclusion perfusion was normal in all animals. Although after LAD ligation perfusion was equally altered in both groups (placebo: $73.5 \pm 1.8\%$, VEGF: $69.2 \pm 4.1\%$, P = NS), 15 days later *VEGF*-treated sheep showed higher perfusion ($78.3 \pm 3.6\%$) than placebo animals ($67 \pm 3.7\%$, $P < 0.05$). The percent change in myocardial perfusion between days 2 and 15 after transfection was $16 \pm 10.8\%$ in *VEGF*-treated sheep and $-8 \pm 3.5\%$ in placebo sheep ($P < 0.05$).

In two animals from each group, the gated study failed as a consequence of frequent premature ventricular contractions. Function data from five animals per group are thus reported. In both groups wall motion scores were similar 2 days post-AMI and showed a tendency to improve 15 days later (placebo: 10.4 ± 1.6 and 7.6 ± 1.2; VEGF: 9.6 ± 2.2 and 6 ± 1.2; P = NS, paired comparisons). Although this tendency was more pronounced in *VEGF*-treated sheep, the differences with placebo did not reach statistical significance ($P < 0.15$, unpaired comparison).

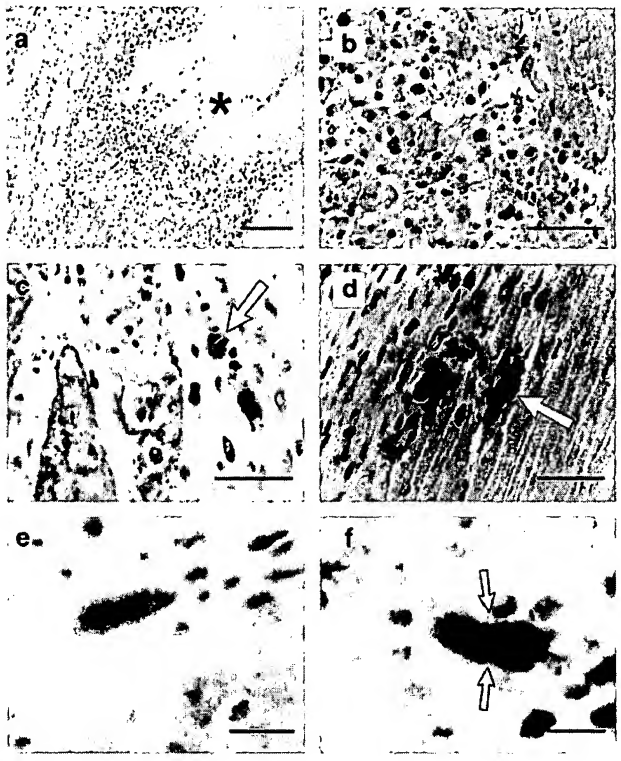

Figure 3 Cardiomyoblasts. (a) In the 10-day-old infarcts numerous small cells with Ki67+ nuclei were found adjacent to the dead myocardium (*). (b) In many of those Ki67+ cells the cytoplasm was stained with an antibody against sarcomeric α-actin. (c and d) Cardiomyoblasts in metaphase (arrows). (e) Anaphase. (f) Late telophase with symmetrical opposite invaginations of the plasma membrane (arrows), suggesting ongoing cytokinesis. (b and d) Nomarski optics. Bars: (a) 200 μm, (b) 100 μm, (c and d) 40 μm, (e) and (f) 20 μm.

Gene expression

In a group of 10 additional sheep with LAD ligation we assessed *VEGF* gene transfer and expression. In all hearts receiving p$VEGF_{165}$ a positive PCR for a specific portion of the plasmid DNA was found 3, 7, 10 and 15 days later ($n = 2$ for each time point). Reverse transcription-polymerase chain reaction (RT-PCR) with specific primers for human VEGF mRNA was positive at 3 days ($n = 2/2$) after p$VEGF_{165}$ administration, but negative at 7, 10 and 15 days postinjection as well as in two placebo animals studied 3 days after injection of empty plasmid (Figure 7a). These negative results confirm specificity of RT-PCR for human (versus endogenous) VEGF mRNA.

In the smooth muscle media layer of some intramyocardial vessels and in the cytoplasm of scattered cardiomyocytes VEGF protein was found by immunohistochemistry at 7, 10 and 15 days after p$VEGF_{165}$ injection (Figure 7b). Consistently negative reactions in control tissue samples established the specificity of the analysis. These results were confirmed by Western blot (Figure 7c). Peak VEGF mass was observed at 10 days post-transfection ($n = 2/2$), but positive reactions were also present at 7 ($n = 2/2$) and 15 ($n = 2/2$) days. Negative results were found at 3 days after p$VEGF_{165}$ injection ($n = 2$) and in placebo-treated hearts ($n = 2$). In infarcted sheep from previous safety studies using equal doses and transfection strategy, we found that Western blot analysis was positive at 35 days ($n = 2/2$) but negative at 450 days ($n = 2$) after transfection (results included in Figure 7c). Western blot for sarcomeric α-actin was employed as internal loading control.

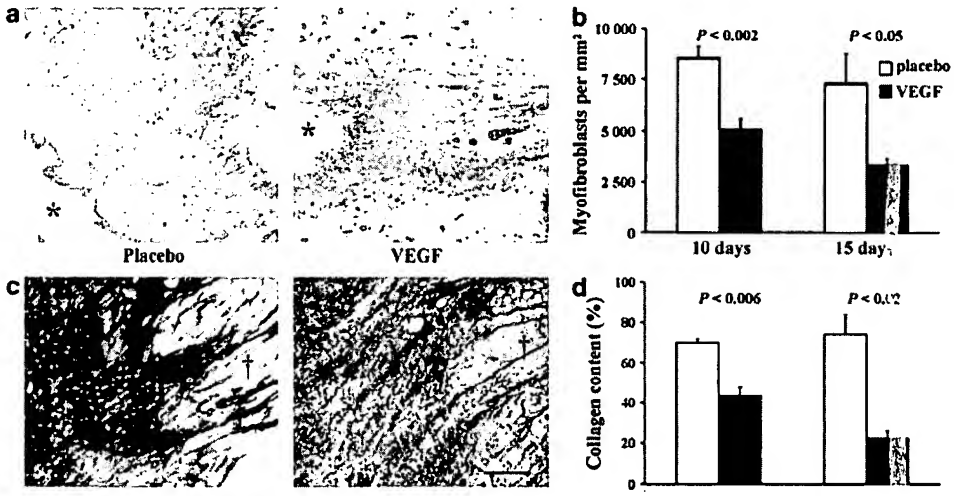

Figure 4 Myofibroblast proliferation and fibrosis. (a) Myocardial tissue sections stained with immunohistochemistry against smooth muscle actin in the zone between dead (*) and surviving myocardium showing decreased myofibroblast proliferation in the *vascular endothelial growth factor (VEGF)*-treated animal. (b) Group results for myofibroblast density at 10 and 15 days after coronary artery ligation. (c) Myocardial tissue sections stained with picrosirius red in the peri-infarct zone adjacent to the viable myocardium (†) showing decreased collagen content in the *VEGF*-treated animal. (d) Group results for collagen content as a percent of total scanned area. Bar: 100 μm.

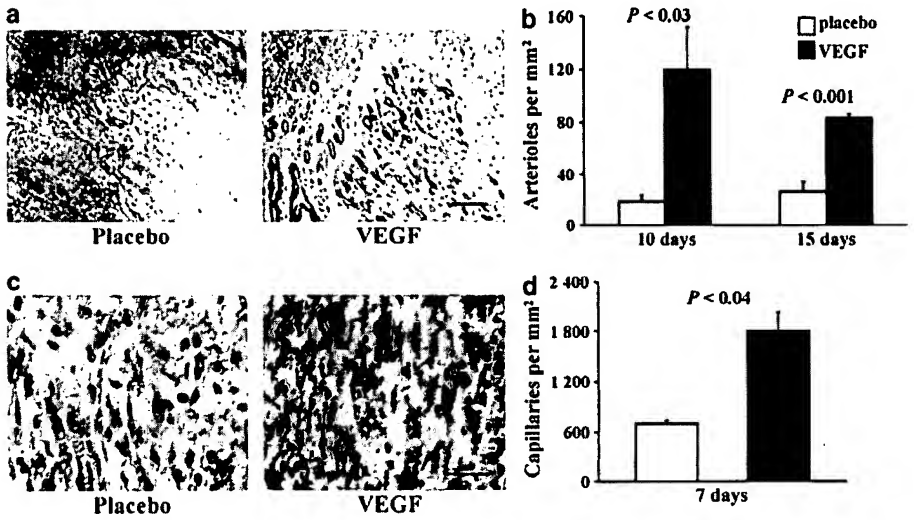

Figure 5 Neovascular proliferation. (a) Immunostaining against smooth muscle actin in the peri-infarct viable myocardium of a placebo- and a *vascular endothelial growth factor (VEGF)*-treated sheep. Arteriolar proliferation is enhanced after *VEGF* gene transfer. Bar: 100 μm. (b) Group results for arteriolar density. (c) Ulex lectin-stained myocardial tissue sections of the zone between dead and viable myocardium at 7 days after coronary artery ligation in a placebo- and a *VEGF*-treated animal. Closely packed capillaries are more abundant after *VEGF* gene transfer. Bar = 50 μm. (d) Group results for capillary density.

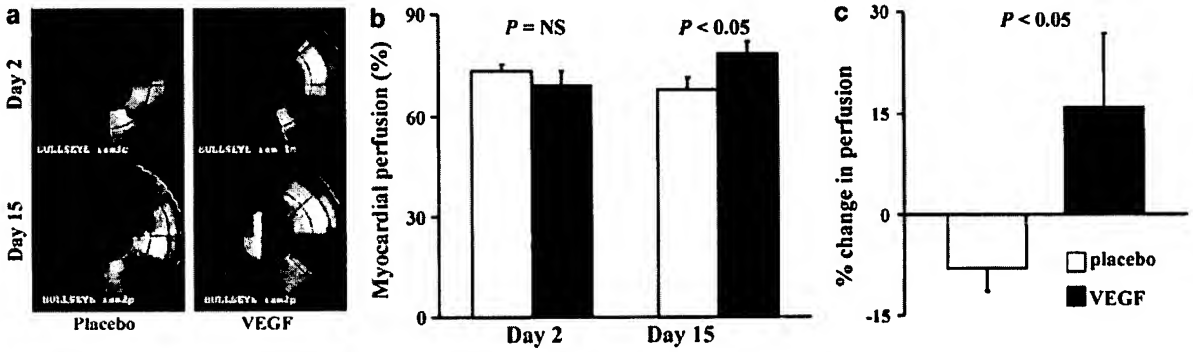

Figure 6 Left ventricular (LV) perfusion (^{99m}Tc-sestamibi SPECT). (a) Polar plots at 2 and 15 days after coronary artery ligation show that while in the placebo-treated animal the left anterior descending (LAD)-dependent perfusion defect increased overtime, it decreased markedly in the *vascular endothelial growth factor (VEGF)*-treated animal. (b) Group analysis shows that the deterioration of perfusion immediately after myocardial infarction was similar in both groups, but 15 days later *VEGF*-treated sheep had significantly higher resting perfusion than placebo animals. (c) The percent change between days 2 and 15 shows an improvement in *VEGF*-treated sheep and a worsening in placebo sheep, the difference being significant.

Immunoassay specificity was confirmed with a standard of human-purified VEGF.

Discussion

Infarct size

The first study on the effect of angiogenic growth factors on AMI was reported more than 10 years ago.[9] Later, VEGF protein was shown to increase myocardial blood flow in porcine hearts,[10] and growth factors were proposed as potential tools for reducing infarct size.[11] On account of the short half-life and potential harmful effect of recombinant growth factors, efforts were oriented towards gene therapy.

To our knowledge, the present study is the first to show in a large mammalian model of AMI that transfecting the heart with the human *VEGF* gene significantly reduces infarct size 15 days after LAD occlusion. Most studies on the effect of transfection of genes encoding for angiogenic growth factors on experimental AMI have been made in rodents[12–17] and only a few in large mammals. Moreover, the latter studies have used a model of chronic ischemia rather than AMI,[18] or assessed variables other than infarct size.[19]

When selecting a large mammalian model of AMI we chose the sheep because, unlike pigs, whose cardiomyocytes have up to 32 nuclei,[1,2] ovine cardiomyocytes have only 1–4 nuclei, thus being more similar to human.[20] Dogs, on the other hand, have extensive innate collaterals, which may influence the size of AMI independently from the tested intervention. This is not the case for ovine coronary circulation, where no collateral vessels would confound the determination of infarct size.[21]

Given that in our study infarct size reduction was evident at 15 days but not at 10 days after LAD ligation, the infarct-limiting effect must have occurred in only

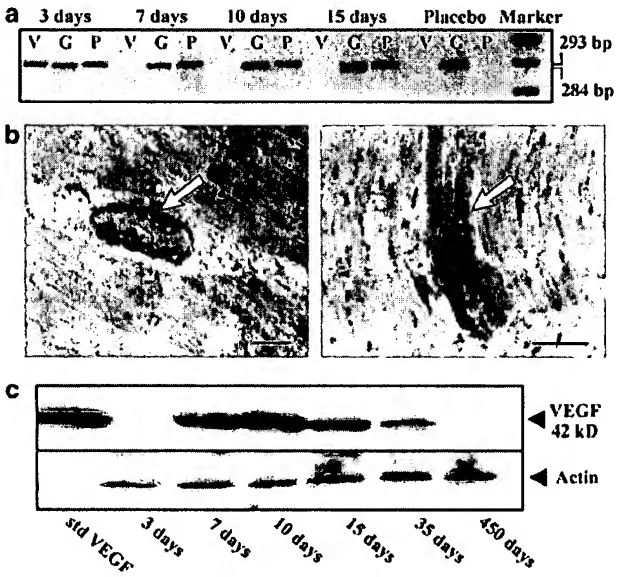

Figure 7 Time course of human vascular endothelial growth factor (VEGF) expression in ovine peri-infarct myocardium. (a) PCR for DNA of plasmid encoding *VEGF* (lane P) showed plasmid presence at all time points and reverse transcriptase-polymerase chain reaction (RT-PCR) for VEGF mRNA (293 bp, lane V) showed gene expression at 3 days after plasmid-mediated *VEGF* gene transfer. Polymerase chain reaction and RT-PCR were negative after 3 days of empty plasmid injection (placebo), whereas mRNA integrity and loading control was verified in all samples (RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 284 bp, lane G). (b) Immunohistochemistry revealed location of VEGF protein (arrows) in the smooth muscle of arterioles (left) and in the cytoplasm of adult cardiomyocytes (right). Bars: 40 μm. (c) Western blot confirmed protein expression results at 7, 10 and 15 days after *VEGF* gene transfer in protocol sheep and at 35 and 450 days in additional sheep (see text). Loading control was assessed with sarcomeric α-actin analysis and specificity was validated with a standard of human-purified VEGF (std VEGF, 42 kDa).

5 days, and VEGF must have acted upon the mechanisms engaged in early infarct expansion, a phenomenon resulting from the interaction of cellular and extracellular post-AMI events that conduct to changes in left ventricular (LV) geometry.[22–24]

Neovascularization

At 7 days after infarction, coincident with the detection of human $VEGF_{165}$ in heart tissue, transfected sheep showed an increase in the neoangiogenesis that normally occurs in the infarct.[12] In addition, $pVEGF_{165}$ induced arteriogenesis, an effect reported in mice[14] and pigs.[25] At 10 and 15 days post-AMI, the number of arterioles in the surviving myocardium immediately adjacent to the infarct was higher in the *VEGF*-transfected group. Improved perfusion resulting from enhanced angio-arteriogenesis may have rescued cardiomyocytes that otherwise would have been lost, and this might be a likely reason for the reduction in infarct size observed later. This assumption is supported by two previous observations: VEGF is essential for cardiomyocyte survival through its effect on maintenance of the capillary bed;[26] and in humans late reperfusion after infarction may benefit remodeling and function.[27]

Fibrosis

Vascular endothelial growth factor transfection modified the pattern of the reparative process. At 10 and 15 days post-AMI, *VEGF*-treated animals exhibited decreased myofibroblast proliferation and collagen in the area between dead and viable myocardium. Although the mechanisms involved were not explored, it can be speculated that VEGF inhibited differentiation of fibroblasts into myofibroblasts and subsequent proliferation, as has been shown in rats with glomerulosclerosis and progressive renal failure, where VEGF reduced renal fibrosis.[28,29] Alternatively, increased angiogenesis occurring at 7 days postinfarction may have replaced or inhibited fibroblast proliferation, as supported by the fact that VEGF enhances experimental wound healing by promoting angiogenesis.[30,31]

Cardiomyogenesis

In addition to neovascularization and antifibrogenesis, VEGF increased the density of a population of small cells located in the area between dead and viable myocardium. Many of them were in the cell cycle, as demonstrated by Ki67 antigen expression, and showed mitotic bodies and occasional cytokinesis. Given their small size, presence of sarcomeric α-actin and connexin-43 and absence of smooth muscle and endothelial cell markers, it can be assumed that they were proliferating cardiomyoblasts. Unfortunately, the lack of commercial antibodies for stem cells markers in sheep prevented to determine if they were originated from resident progenitors[32,33] or cardiac stem cells.[34,35] In addition, the MDR-1-positive reaction cannot be considered as a proof for undifferentiated phenotype, since MDR-1 is also found in ischemic adult cardiomyocytes.[6] The presence of these cells was transient and they were not observed at 15 days post-AMI.

As has been reported for human AMI,[3] at 10 days after LAD occlusion all sheep showed mitotic activity of adult cardiomyocytes in the peri-infarct area. This phenomenon was markedly enhanced by *VEGF* gene transfer, confirming our previous results.[1] With the present data we cannot establish the mechanisms of this effect. The increased mitotic activity in adult myocytes and cardiomyoblasts can be due to VEGF-induced improved perfusion, as has been hypothesized for implantation of bone marrow stem cells[36,37] or skeletal myoblasts transfected with the *VEGF* gene,[38] or to a direct effect of VEGF.[1]

On account that growth factors may display pleiotropic actions,[39] the possibility that VEGF acted on diverse targets should be considered. In fact, in pigs with chronic myocardial ischemia, *VEGF* gene transfer induces not only angio-arteriogenesis in ischemic territories[25] but also increases the mitotic index of adult cardiomyocytes in ischemic and non-ischemic myocardium.[1] Moreover, the evidence that in this pig model VEGF induces hyperplasia of adult cardiomyocytes[2] was the reason that encouraged us to conduct the present study. Furthermore, a recent report in a similar model shows that transfer of human *FGF-5* gene increases cardiomyocyte mitotic index.[40] On these bases, cardiomyoblast proliferation and entrance in mitosis of adult cardiomyocytes, with evidence of occasional cytokinesis, may have also contributed to decreased infarct size.

Left ventricular function and perfusion

Vascular endothelial growth factor-treated animals showed a tendency for improved ventricular motility. The lack of significance could be due to the short time elapsed between the infarct-limiting effect and the moment of evaluation. If peri-infarct ischemia existed, it is reasonable to speculate that angio-arteriogenesis induced by *VEGF* gene transfer had not yet resulted in a degree of functional recovery high enough to yield significant differences with respect to placebo group. Contrarily, the effect of VEGF on myocardial perfusion was consistent with infarct size results. In effect, given that SPECT studies were carried out at rest, the higher perfusion values in *VEGF*-treated sheep represent larger amount of viable myocardium rather than augmented blood flow.

Gene expression

Presence of VEGF protein (confirmed by immunohistochemistry and Western blot) far beyond the disappearance of mRNA expression is interesting. Similar time lags between both phenomena were reported by Couffinhal *et al*.[41] in mice with hindlimb ischemia and by us in pigs with chronic myocardial ischemia.[2,25] The most likely explanation is the known ability of the secreted protein to remain bound to VEGF-binding sites for long periods.[42]

Conclusion

In adult sheep human $VEGF_{165}$ gene transfer reduces infarct size at 15 days after acute coronary artery occlusion. The mechanisms involved include neovascular formation, reduced fibrosis and increased cardiomyocyte regeneration. Vascular endothelial growth factor gene transfer may thus represent an approach for treatment of AMI and prevention of LV remodeling.

Materials and methods

Plasmid construct

The eukaryotic expression vector (p$VEGF_{165}$, deposited as pBSVEK3 at *Deutsche Sammlung von Mikroorganismen und Zellkulturen*, accession number DSM 14 346) is a 3930 bp plasmid that includes the human $VEGF_{165}$ coding gene, transcriptionally regulated by the cytomegalovirus promoter/enhancer, and a SV40 poly-A terminator. The placebo plasmid (pLUSK3, 3317 bp, accession number DSM 14 384) is obtained from p$VEGF_{165}$, by excision of the human $VEGF_{165}$ coding gene. Preparation procedures, purification and quality control analyses were performed under GMP conditions (Bio Sidus, Buenos Aires, Argentina).

Surgical preparation and experimental protocols

Twenty six Corriedale male sheep aged 12–16 months and weighing 23 ± 0.4 kg were operated. All procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Following premedication with acepromazine maleate (5 mg, i.m.), anesthesia was induced with intravenous sodium thiopental (20 mg/kg) and maintained with 1.5% halothane in pure oxygen under mechanical ventilation (Neumovent, Córdoba, Argentina). After a sterile thoracotomy at the 4th intercostal space, the LAD artery was ligated at its distal third. The second diagonal branch was also ligated at a point in line with the LAD ligature.[21] This resulted in an apical infarct comprising approximately 20% of the LV mass. To reduce the incidence of ventricular arrhythmias, lidocaine (three bolus injections of 2 mg each and a 2 mg/kg infusion), amiodarone (150 mg infusion in 2 h) and atenolol (2 mg) were administered. After 1 h, 3.8 mg of p$VEGF_{165}$ or placebo plasmid was injected intramyocardially in ten 0.2 ml aliquots. The nature of the injectates was kept blind until the end of data analysis. Injections were distributed along the normoperfused tissue 10–15 mm distant from the border of the ischemic zone. This zone was readily recognized by the presence of cyanosis and dyskinesia.

Sheep were killed at two different time points: 12 (*VEGF* treated: $n=6$, placebo: $n=6$) at 10 days and 14 (seven per group) at 15 days post-AMI. The sheep were killed with an overdose of sodium thiopental followed by a bolus of potassium chloride. For angiogenesis assessment, eight additional sheep (*VEGF* treated: $n=4$; placebo: $n=4$) prepared and treated as previously described were killed at 7 days post-AMI. For transgene expression studies we employed 10 additional sheep with AMI: eight receiving p$VEGF_{165}$, killed at 3, 7, 10 and 15 days ($n=2$ for each time point), and two receiving placebo, killed at 3 days post-AMI. Five myocardial tissue specimens from each heart were removed from the peri-infarct area, snap frozen in liquid nitrogen and stored at −70°C for PCR, RT-PCR and Western blot, or fixed in 10% buffered formaldehyde for immunohistochemistry.

For assessing if VEGF-induced cardiomyogenesis could also take place in non-infarcted hearts, 12 additional sheep without coronary ligation were randomized to blindly receive p$VEGF_{165}$ ($n=6$) or empty plasmid ($n=6$) in the same doses. Injections were distributed in the anterolateral LV wall, and sheep were killed 10 days later for histological analysis of the injected myocardium.

Left ventricular function and perfusion

In the group of 14 sheep killed at 15 days post-infarction, myocardial function and perfusion were studied at normal, pre-LAD occlusion state, 2 days post-AMI and just before killing. Studies were carried out in an ADAC Vertex Dual Detector Camera System (Milpitas, CA, USA) using ^{99m}Tc-sestamibi (G-SPECT). The sestamibi injection was carried out at conscious, resting condition 2 h before acquisition.

Regional wall motion was visually evaluated by two independent observers using the 20 segments model[43] and a motion score in which 0 corresponds to normokinesis, 1 to mild hypokinesis, 2 to moderate hypokinesis, and 3 to akinesia and dyskinesia. The final score at each experimental condition results from the sum of the individual segmental scores. Given the reported inaccuracy of the QGS software to detect the endocardial surface in small hearts,[44] the volume and ejection fraction data were disregarded.

For LV perfusion, the circumferential count profiles (polar plots) determining the number of counts per segment were analyzed in each study, as previously described.[25] The analysis of the results was focused on the zones corresponding to the occluded LAD territory: apical and medial segments of the anterior and septal

sectors, and apical segment of the infero-septal sector. Percent change in myocardial perfusion was calculated as ((perfusion at day 15−perfusion at day 2)/perfusion at day 2) × 100.

Infarct size measurement

In animals killed at 10 ($n=12$) and 15 ($n=14$) days, the LV was opened through an incision parallel to the posterior interventricular sulcus and extended flat before fixation. Digital photographs were obtained for image processing (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD, USA) to determine LV and infarct areas. Infarct size was expressed as percent total LV area.

Histological and immunohistochemical studies

The whole heart was immersed in 10% buffered formaldehyde. After at least 48 h fixation, 5 mm thickness transversal slices of the whole LV wall, including the interventricular septum, were obtained at the level of the infarct center and at 10 and 20 mm above and below. The tissue slices were divided in four blocks that were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin–eosin, Masson's trichrome and picrosirius red. For immunohistochemical studies tissue sections were deparaffinized and brought to phosphate-buffered saline, pH 7.2. After blocking endogenous peroxidase with 3% H_2O_2 in methanol and antigen retrieval pretreatment with citrate buffer in a microwave oven, the slides were incubated 1 h with rabbit anti-human $VEGF_{165}$ polyclonal antibody (BioGenex, San Ramon, CA, USA) or specific monoclonal antibodies against the Ki67 antigen (Novocastra, Newcastle upon Tyne, UK) and smooth muscle actin (BioGenex), and post-treated with biotinilated anti-mouse and anti-rabbit immunoglobulin antiserums (Multilink, Biogenex), followed by peroxidase-labeled avidin, and revealed with AEC as chromogen. In addition, tissue sections were double stained with monoclonal antibodies against the Ki67 antigen and sarcomeric α-actin (Dako, Carpinteria, CA, USA), connexin-43 (Zymed, San Francisco, CA, USA), sca-1 (Sigma, St Louis, MO, USA), c-kit (Biogenex) and the *MDR-1*-gene-encoded P170 glycoprotein (clone C494, Signet Laboratories, Dedham, MA, USA; clone MDR-88, Biogenex, San Ramon, CA, USA) as previously described.[1,6] Endothelial cells were identified by means of biotinilated *Ulex europaeus* lectin (Vector, Burlingame, CA, USA).[7]

Collection of quantitative data was carried out in two different zones. Adult cardiomyocyte mitosis and arteriolar density (vessels with smooth muscle actin-positive wall and measuring 50 μm or less in diameter) were determined in an area comprising 10 rows of myocytes of the surviving myocardium adjacent to the infarct border (2.3 ± 0.2 mm^2). Adult cardiomyocytes were identified by their size, shape, nuclear morphology and presence of cross-striations and cytoplasmic sarcomeric α-actin. The second zone corresponded to the area present between the remnant of coagulation necrosis tissue and the viable myocardium (2.1 ± 0.1 mm^2). At that level, cardiomyoblast proliferation, angiogenesis, myofibroblast proliferation (smooth muscle actin-positive cells) and fibrosis (red areas after picrosirius red staining) were determined.

Adult cardiomyocyte mitotic index was calculated according to previously described procedures for pig[1] and human[3] hearts. Number of mitosis was expressed respective to the number of CMN in the examined area. Other data (capillaries, arterioles, cardiomyoblasts and myofibroblasts densities) were expressed as number of structures per mm^2.

Gene expression

Presence of $pVEGF_{165}$ and expression of human $VEGF_{165}$ were assessed by PCR, RT-PCR and immunohistochemistry as above mentioned. In addition, human $VEGF_{165}$ protein mass was studied with Western blot at 3, 7, 10 and 15 days after *VEGF* gene transfer. Presence of human VEGF protein in ovine myocardium at longer times after transfection (35 and 450 days) was studied on specimens of infarcted sheep belonging to previous safety protocols where we used the same doses and transfection strategy as in the present protocol.

PCR: Total DNA was isolated (Qiagen, Hilden, Germany) and quantitated ($A_{260/280}$ nm spectrophotometry). Amplification was done using *Taq* polimerase (Perkin-Elmer, Boston, MA, USA) and previously reported *ad hoc* primers.[1]

RT-PCR: Total RNA was isolated (Trizol reagent, Gibco BRL, Grand Island, NY, USA), treated with DNAse I (Promega, Madison, WI, USA), quantitated and reverse transcripted (random hexamers, Perkin-Elmer). Non-competitive amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed to demonstrate mRNA integrity. To discard false-positive results, RT-PCR of isolated RNA from $pVEGF_{165}$-treated hearts was performed omitting the RT reaction.

Western blot: tissue samples weighing 0.1–0.2 g were homogenized in 1 ml lysis buffer, containing 10 mmol/l Tris HCl, pH 7.6, 100 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/l EDTA, pH 8, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride. Samples were centrifuged (10 000 *g* for 20 min) and the supernatants analyzed for protein concentration using a Bradford assay (Bio-Rad, CA, USA). Sample proteins (100 μg) were resolved under non-reducing conditions on a 12% SDS-polyacrylamide gel. Immunoblotting was performed using a monoclonal antibody against human $VEGF_{165}$ (MAB 293, R&D Systems, MN, USA) at a dilution of 1:100 in non-fat milk/Tris buffer. The membrane was subsequently probed with a secondary anti-mouse antibody conjugated to horseradish peroxidase (P260 Dako, CA, USA) at a dilution of 1:1000 and developed with chemiluminescence (ECL, RPN 2106, Amersham, IL, USA). The membrane was then exposed to X-ray film (BioMax ML, Kodax, NY, USA). A standard of human purified $VEGF_{165}$ (293-VE, R&D Systems) was used as positive control. To exclude the influence of fibrosis on myocyte proteins, immunoblotting for sarcomeric α-actin was performed as an internal loading control.

Statistics

Left ventricular perfusion, infarct size and morphometric results (except capillary density) were analyzed with Student's *t*-test for unpaired data. For capillary density, a non-parametric procedure (Mann–Whitney rank-sum test) was employed due to the small sample size ($n=4$ per group). Left ventricular function was analyzed with non-parametric two-way ANOVA (Friedman's test).

$P<0.05$ was considered to indicate statistical significance. Results are expressed as mean ± s.e.m.

Acknowledgements

This work was supported by grants from The René G Favaloro University Foundation and by grant PID #268 from the Argentine National Agency for Scientific and Technological Promotion (ANPCyT), Secretariat for Technology, Science and Productive Innovation (SeTCIP). Drs Crottogini and Laguens are established Investigators of National Council for Scientific and Technical Investigations (CONICET). We thank technicians Julio Martínez, Fabián Gauna and Marcela Álvarez, veterinarians María Besansón, Pedro Iguain and Marta Tealdo and animal house personnel Juan Ocampo, Osvaldo Sosa and Juan Mansilla.

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